Behavioural and Pharmacological Studies on Learning and Memory in the Honeybee, *Apis mellifera L.*

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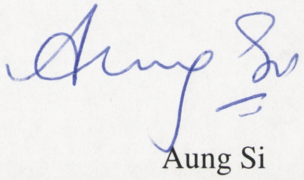
This thesis is submitted in complete fulfilment of the requirements for the degree of Doctor of Philosophy, and does not exceed 100,000 words. Work presented herein is the original work of the author, except where otherwise acknowledged.



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Abstract

The honeybee (*Apis mellifera* L) was used as a model organism to study various aspects of learning and memory, using a range of experimental paradigms, behavioural assays and pharmacological interventions. First, the Proboscis Extension Reflex (PER) paradigm was investigated using the glutamergic drugs L-*trans*-2,4-PDC, memantine and MK-801 to determine the involvement of glutamate in learning and memory processes in the honeybee. L-*trans*-2,4-PDC and MK-801, in spite of differing modes of action, reduced the recall of long-term olfactory associative memory, while memantine was found to restore recall in L-*trans*-2,4-PDC-treated bees. These experiments provide strong evidence in favour of an important role for glutamate in memory-related processes in the honeybee. Second, the dance behaviour of foraging bees was studied, both by training them to fly through narrow tunnels, and under field conditions. The tunnel bees showed that while the honeybee visual system is robust to large variations in contrast and spatial frequency, their dances differ markedly from bees flying out in the open. The large proportion of non-waggle loops observed in the dances of tunnel bees could be the result of conflict between the visual information being provided by the tunnel, and other, yet unknown cues: this conflict could also be the result of using bees of differing (and unknown) ages. The bees in the field study were controlled for age, and their dances observed for most of their foraging careers. The probability of foraging and frequency of visits to the experimental feeder

were found to increase with age, as was the probability of dancing. Caffeine was also administered to some bees in this experiment, and was found to chronically depress the probability of foraging, and acutely reduce visit frequency. Caffeine also increased visit frequency in a Delayed Match-to-Sample task, and improve overall performance. Finally, real-time RT-PCR was used to monitor gene expression changes brought about by some of the above drug treatments. The technique was found to be a reliable way of determining the up- or downregulation of known, individual genes.

1. Chapter 1: Introduction

Ever since the discovery of the dance language of the honeybee *Apis mellifera* by Karl von Frisch and his students in the first half of the 20th century, an impressive amount of research has been carried out on virtually every aspect of honeybee behaviour. That research focused not only on the dance language itself (Dyer, 2002), but also on the manner in which honeybees utilize this unique form of communication in their everyday lives (Seeley, 1995). More recently, honeybees have been shown to be capable of a variety of cognitive feats, previously considered to be the exclusive domain of vertebrates. Not only do bees readily exhibit simple forms of learning such as classical conditioning and habituation(Kuwabara, 1957; Bitterman *et al.*, 1983), they can also be trained to perform much more complex tasks, such as the navigation of a maze to reach a food reward (either by learning a route, or by following symbolic visual instructions (Zhang *et al.*, 1996), perform delayed match-to-sample (DMTS) tasks (Giurfa *et al.*, 2001), memorize and recall groups of visual stimuli to retrace old foraging paths (Zhang *et al.*, 1999), and to categorize groups of objects on the basis of general features (Zhang *et al.*, in press). The ease with which honeybees can be trained, as well as the species' large behavioural repertoire, make the honeybee an ideal model organism for studies on insect behaviour in general, and learning and memory in particular. Moreover, the relatively small and highly

compartmentalized honeybee brain (Hammer and Menzel, 1995) facilitates the investigation of the physiological and biochemical correlates of the various stages of learning and memory. Consequently, investigations such as the role of various neurotransmitters in the honeybee central nervous system (CNS), the mode of action of memory-enhancing and memory-impairing pharmaceutical agents, and the resulting gene expression changes accompanying such drug treatments are made possible.

This thesis aims to investigate various facets of learning and memory in the honeybee, making use of a number of behavioural assays and pharmacological interventions. First, a simple learning paradigm, in conjunction with drugs of known action, is used to determine if glutamate plays a role in associative memory-related pathways. A comparative study of two different simple learning paradigms is also carried out, to establish the usefulness of including punishing stimuli in associative learning training protocols. Next, the waggle dance of honeybees is examined, to bring to light any effects of age on various parameters of this complex behaviour. The effects of caffeine on the waggle dance, as well as a relatively complex learning task will also be looked into, to determine this drug's effects on arousal and learning.

1.1 Simple learning in the honeybee

Honeybees can be easily trained to exhibit simple forms of learning, such as habituation and classical conditioning. *Habituation* is simply the process by which an animal learns to ignore a stimulus, that it is repeatedly exposed to

(Kandel *et al.*, 2000). Young honeybees have a well-developed ability to habituate to a repeated, non-rewarding stimulus (Bicker and Hahnlein, 1994), and this ability has been found to decrease with age (Guez, 2001; Guez *et al.*, 2003). *Classical conditioning*, on the other hand, requires an animal to associate one type of stimulus (the conditioned stimulus, or CS) with another (the unconditioned stimulus, or US) (Kandel *et al.*, 2000). Honeybees can be trained to associate both olfactory and visual conditioned stimuli with an unconditioned stimulus such as a reward of sugar water. A training protocol for olfactory conditioning in the honeybee was first developed by Kuwabara (1957), and later modified by Bitterman *et al.* (1983), who, by means of repeated paired associations of odour (CS) and sucrose solution (US), were able to rapidly train forager honeybees to produce a proboscis-extension response (PER). The extension of the proboscis is the normal response of a honeybee when its antennae or mouthparts are stimulated with sucrose solution. Following training with the Bitterman *et al.* (1983) protocol, however, exposure to only the conditioning odour would lead to a PER. Both habituation and the PER paradigm have been extensively investigated in recent years, in an attempt to better understand their underlying physiological and biochemical substrates (see Table 1).

Adult forager bees (*i.e.* free-flying bees) can also be trained to perform visual association tasks with the help of a Y-maze (Srinivasan and Lehrer, 1988). Bees flying through the base of the Y-maze are confronted with two competing visual stimuli (colours, shapes, gratings, *etc.*), only one of which conceals a sugar reward behind it. Naïve bees are forced to choose between the two stimuli, and, after repeated visits, learn to associate the correct stimulus with the reward.

STUDY	PARADIGM	TYPE OF INTERVENTION	MODE OF ACTION	EFFECT ON MEMORY
Wittstock *et al.*, 1993	Two-trial olfactory conditioning	Cycloheximide	Protein synthesis inhibitor	None
Gauthier *et al.*, 1994	One-trial olfactory conditioning	Scopalamine	mAch receptor antagonist	Impairment of retrieval of STM
Lozano *et al.*, 1996	One-trial olfactory conditioning	Mecamylamine into brain haemolymph	nAch receptor antagonist	Impairment of acquisition and retrieval of STM
Müller, 1996	One- or three-trial olfactory conditioning	NOArg, L-NAME	Nitric oxide synthase inhibitors	Impairment of three-trial LTM
Hammer and Menzel, 1998	Eight-trial olfactory conditioning	Pairing of CS with octopamine injection into MB	Neurotramsmitter	Induces conditioning
Lozano and Gauthier, 1998	One-trial olfactory conditioning	Atropine, pirenzepine	mAch receptor antagonists	Atropine impairs retrieval of STM
Morgan *et al.*, 1998	One-trial olfactory conditioning	Queen removal	unknown	Age-dependant impairment of STM
Wüstenberg *et al.*, 1998	Three-trial olfactory conditioning	Actinomycin-D, anisomycin	Transcription, translation blocker respectively	Impairment of 4d memory, 2-3d memory unaffected by both drugs
Fiala *et al.*, 1999	Three-trial olfactory conditioning	PKA antisense S-ODN	Knockdown of PKA activity	Impairment of LTM
Menzel *et al.*, 1999	Four-trial olfactory conditioning, sensitization	Reserpine, dopamine, octopamine, serotonin	Reserpine depletes brain biogenic amines; dopamine, octopamine, serotonin are neurotransmitters	Reserpine, impaired STM & sensitization; octopamine rescues acquisition of STM; serotonin impairs acquisition of STM
Maleszka *et al.*, 2000	Three-trial olfactory conditioning	L-*t*-PDC	Glutamate transporter inhibitor	Impairs LTM
Lozano *et al.*, 2001	One-trial olfactory conditioning	Mecamylamine and scopolamine into AL and C of MB	nAch receptor antagonist, mAch receptor antagonist respectively	Impairment of STM following injection into AL
Guez *et al.*, 2001	Habituation of the PER	Imidacloprid	nAch receptor agonist	Impairs habituation in ≤7d-old bees, enhances in >7d-old bees
Lambin *et al.*, 2001	Habituation of the PER	Imidacloprid	nAch receptor agonist	(Increases gustatory threshold for sucrose solution), enhances habituation in older bees
Maleszka and Helliwell, 2001	One-trial olfactory conditioning	Juvenile hormone	Accelerates developmental processes (?)	Improvement of STM retention
Müller and Hildebrandt, 2002	Habituation of the PER	KT5720, Rp-BrcAMPS, BrcAMP, caged cAMP	KT5720 and Rp-BrcAMPS are PKA inhibitors; BrcAMP and caged cAMP are PKA activators	KT5720, Rp-BrcAMPS impair habituation; BrcAMP, caged cAMP improve habituation
Decourtye *et al.*, 2003	Three-trial olfactory conditioning	Imidacloprid and 5-hydroxy imidacloprid	nAch receptor agonists	Impairment of memory, winter bees need larger doses than summer bees

Farooqui *et al.*, 2003	Six-trial olfactory conditioning	Mianserin, AmOAR dsRNA	Octopamine receptor antagonist, silencing of octopamine receptor expression respectively	Impairment of acquisition and recall of STM and LTM
Guez *et al.*, 2003	Habituation of the PER	Imidacloprid metabolites olefin, 5-hydroxy imidacloprid	nAchR agonists	Olefin impairs habituation in 7 & 8d-old bees, 5-hydroxy imidacloprid improves habituation in 8d-old bees
Müller *et al.*, 2003	Four-trial olfactory conditioning	Procaine, lidocaine	Local anaesthetics	Procaine impairs acquisition and consolidation of LTM
Tautz *et al*, 2003	One-trial olfactory conditioning	Temperature at pupal development controlled: 32°C, 34.5°C and 36°C	Influence on anatomical and physiological development (?)	36°C treatment enhances STM
Decourtye *et al.*, 2004	Olfactory conditioning of free-flying bees, three-trial olfactory conditioning	Imidacloprid	nAch receptor agonist	Impairs olfactory learning, acquisition of conditioning

Table 1. Recent studies on learning and memory in honeybees involving pharmacological and physical interventions. AL: antennal lobe, AmOAR: honeybee octopamine receptor, C: calyx, cAMP: cyclic adenosine monophosphate, CS: conditioned stimulus, dsRNA: double-stranded ribonucleic acid, mAch: muscarinic acetylcholine receptor, MB: mushroom body, nAch: nicotinic acetylcholine receptor, PER: proboscis extension reflex, PKA: protein kinase A, Rp-8-BrcAMPS: Rp isomer 8-bromo-adenosine-3,5-cyclic monophosphorothioate, S-ODN: phosphorothioate-modified oligodeoxynucleotides, STM: short term (~1h) memory, L-NAME: N-nitro-L-arginine methyl ester, LTM: long-term (~24h) memory, L-*t*-PDC: L-*trans*-2,4-pyrrolidine decarboxylate, NOArg: N-nitro-L-arginine.

1.1.1 Stages in the learning and recall processes

From a functional experimental perspective, the memory process can be divided into two main stages: *encoding*, which refers to the process of acquiring information or placing it into memory, and *retrieval* or *recall*, which is the process of recovering previously encoded information (Brown and Craik, 2000). The encoding step can be further subdivided into 1) an *acquisition* stage, which occurs following a pairing of CS and US, and in mammals requires the NMDA receptor (see Section 1.2.1), the enzyme protein kinase A (PKA) and an intact hippocampus (Abel and Lattal, 2001), and 2) a *consolidation* stage, which in mammals requires new mRNA and protein synthesis mediated by the transcription factor CREB (Tonegawa *et al.*, 2003). While newly-learnt information is sensitive to disruption immediately following acquisition (Abel and Lattal, 2001), a period of consolidation lasting for hours or even days allows the memory to be strengthened (Brown, 2002), and later transferred to other brain regions (Bontempi *et al.*, 1999). From a human cognitive perspective, the effective encoding of LTM requires "paying attention" to the new information, the placing of that information in a complex, elaborate and meaningful context, and the spacing out over time of any subsequent rehearsals of that information (Brown and Craik, 2000). Retrieval, on the other hand, does not seem to require NMDA receptors, PKA, PKC or protein synthesis, all of which are crucial in the encoding step (Abel and Lattal, 2001). Instead, normally functioning hippocampal CA3 cells seem to be the key to unimpaired memory recall (Tonegawa *et al.*, 2003). Human psychophysical studies have shown repeatedly, that the retrieval of learnt information depends heavily on a person's mental state, as well as the context

within which that information has been learnt, *i.e.*, successful retrieval depends on the similarity between retrieval and encoding cues (Brown and Craik, 2000).

Several memory phases, ranging from early short-term memory (eSTM) to late long-term memory (lLTM), have been distinguished in the honeybee, based on the results of behavioural and pharmacological studies (Menzel, 2001). The early form of STM is induced merely a few seconds following a single pairing of CS and US; this is characterised by the convergence of excitation in the CS and US pathways. Late STM (lSTM) is the next stage in the memory formation pathway, lasting up to several minutes after a single pairing or trial, and can also be considered, in combination with the eSTM to be a component of the honeybee's working memory phase (Menzel, 1999). At the end of this stage, the memory is said to have become more specific (context dependant), and consolidated (resistant to extinction, conflicting information and elapsed time). In the case of multiple trial learning, however, lSTM is attained very quickly, and appears immediately on trial repetition (Menzel and Sugawa, 1986). While single-trial conditioning alone cannot induce long-term memory (LTM) in honeybees, multiple-trial conditioning is able to do so, and is accompanied by a profound prolongation of PKA activation in the antennal lobes (Müller, 2000). Interestingly, the artificial enhancement of PKA activity in single-trial conditioned honeybees mimics the effect of multiple-trial conditioning, and induces LTM.

It seems pertinent to mention at this point the distinction between STM and working memory (WM). While the two terms are often used interchangeably in the literature (Baddeley, 2000), WM refers more to the functional role of STM in the context of holding and manipulating learnt information during the

performance of complex cognitive tasks (Baddeley and Hitch, 1974; Logie, 1996). The decline in performance in a DMTS task (see Fig. 5.1), brought about by progressively increasing the delay between the sample and choice stimuli (Zhang *et al.*, in prep), can therefore be attributed to a decrement in the retention of the sample stimulus in STM. In any event, the distinction between (or even the existence of) the two forms of memory remains a divisive issue in human psychology (Logie, 1996), and is beyond the scope of this thesis.

The transition between STM to the next form of memory, mid-term memory (MTM), takes up to several minutes, and is accompanied by a first wave of protein kinase C (PKC) activity in the antennal lobes of the conditioned individual. Again, this is brought about only by multiple learning trials; single-trial conditioning has no effect on PKC levels (Grünbaum and Müller, 1998). The MTM phase lasts several hours, and retention during this phase can be blocked by applying proteases to the whole brain (Menzel, 2001).

LTM can be further subdivided into two phases: early LTM (eLTM and late LTM (lLTM). Whereas eLTM is characterised by retention of the learnt association 1-2 days after conditioning, lLTM only appears after an interval $\geq$3 days. The distinction is based on the dependence of the two phases on protein synthesis: memory retention in the eLTM phase is unaffected by protein synthesis inhibition, while the same in the lLTM phase is (Menzel, 2001).

For the purpose of clarity, however, only two memory phases are named during the course of this thesis. Chapter 2 deals with two memory types, namely the memory retained one hour after one-trial associative learning (short-term memory, or STM), and the memory retained 24 hours after three-trial learning

(long-term memory, or LTM). Chapter 5 describes a DMTS experiment, where honeybees are required to store a visual stimulus (the Sample) in their STM, and then choose a 'matching' stimulus from two choices following a time delay (a few seconds). This is followed by a Y-maze experiment, where honeybees must learn to associate a reward with a single 'correct' stimulus, and store this stimulus in their LTM (a few hours). These two memory phases were chosen, as it was of interest to compare the effects of previously untested (on honeybees) drugs on two distinct stages of memory. The remaining, transitory phases of memory were not considered to be relevant to the present study.

1.2 Glutamate and long-term memory

Although L-glutamate has long been known to be the major excitatory neurotransmitter in the mammalian CNS (Danbolt, 2001) its function in the insect brain remains unclear. L-glutamate has been found to play a vital role in normal CNS development in vertebrates, not only bringing about long-term potentiation (Durand *et al.*, 1996), synapse elimination and functional synapse induction (Rabacchi *et al.*, 1992), but also modulating neuronal migration, the outgrowth of neuronal processes (Lipton and Kater, 1989) and the activity of other neurotransmitters, such as GABA (Van den Pol *et al.*, 1998).

1.2.1 The NMDA glutamate receptor in learning and memory

Glutamate receptors can be divided into two main subtypes: ionotropic, which are coupled directly to membrane ion channels, and metabotropic, which are coupled to G proteins (Lipton and Rosenberg, 1994). The ionotropic receptors can be further subdivided into three major types based on their selective agonists: N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionate (AMPA) and kainate. It is the first of these, namely the NMDA receptors (NMDAR), which are considered to be the classic learning and memory receptors in the vertebrate CNS.

The NMDAR is coupled to an ion channel with relatively slow kinetics, but which is highly permeable to Ca^{+2} ions, in addition to Na^{+} and K^{+} (Riedel *et al.*, 2003). The channel is normally blocked in its resting state by an Mg^{+2} ion, which is bound to a voltage-dependant site, and is released from the channel by a postsynaptic depolarisation (brought about by NMDA or L-glutamate). The need for the simultaneous presence of a depolarisation and a ligand (*i.e.* L-glutamate *in vivo*) makes the NMDAR the prototypical coincidence detector in the CNS (Kandel *et al.*, 2000). Research on the involvement of glutamate (and in particular, NMDA receptors) in learning and memory has largely focussed on vertebrate models, such as the rat, the mouse, primates and the goldfish (Riedel *et al.*, 2003). NMDAR involvement has been demonstrated in these species in various types of learning, such as spatial learning, fear conditioning, inhibitory avoidance learning and olfactory and taste memories; the administration of NMDAR antagonists, such as MK801 and AP5, generally has the effect of impairing memory (Riedel *et al.*, 2003).

1.2.2 Glutamate uptake

The release of glutamate at a normal glutamergic synapse is usually followed by the rapid reuptake of the neurotransmitter into the cells (neural and glial) surrounding the synaptic cleft (Danbolt, 2001). It is crucial that a normal, low extracellular concentration of glutamate be maintained constantly, as excessive stimulation of glutamate receptors may prove harmful to the postsynaptic neuron (Olney and Ho, 1970; Olney, 1990). There are no known extracellular enzymes that could metabolise any excess glutamate within the synaptic cleft, and the passive diffusion of glutamate out of the cleft would be much too slow in the case of large synapses (*e.g.* Rossi *et al.*, 1995). Consequently, glutamate uptake is the only mechanism which maintains the low extracellular glutamate levels that allow a high signal-to-noise ratio in synaptic transmission (Logan and Snyder, 1972; Danbolt, 2001), as well as prevent the detrimental effects of excessive NMDAR stimulation (excitotoxicity) (Lipton and Rosenberg, 1994).

Glutamate uptake in vertebrates is accomplished by means of glutamate transporter proteins, which use the electrochemical gradients across the plasma membranes as driving forces for uptake (Kanner and Sharon, 1978). The activity of these transporters is high enough to even protect neurons that have been artificially exposed to elevated levels of extracellular glutamate *in vitro* for a long period of time (Garthwaite *et al.*, 1992). On the other hand, defective glutamate uptake due to ischemia, for instance, would lead to a subsequent weakening of electrochemical gradients and a very rapid build up of extracellular glutamate, overexcitation of postsynaptic NMDAR, increased Ca^{+2} and Na^{+} influx, and

possible cell injury or death (Lipton, 1999). This can be mediated by the abnormally high activation of intracellular enzyme systems, such as Protein Kinase C, phospholipases, endonucleases and proteases (Lipton and Rosenberg, 1994). It is for this reason that glutamate has been implicated in a wide range of chronic neurodegenerative diseases, including Huntington's Disease, Amyotrophic Lateral Sclerosis and Alzheimer's Disease (Meldrum and Garthwaite, 1990).

1.2.3 Glutamate and the insect CNS

While L-glutamate appears to be the major excitatory transmitter in some insect neuromuscular junctions, its function in the CNS remains unclear. No species has been shown to exhibit the range of agonist and antagonist affinities and ion channel properties described for the vertebrate NMDAR: glutamate can mediate both excitation and inhibition in locusts (Giles and Usherwood, 1985), but insects in general are quite insensitive to NMDA (Glantz and Pfeiffer-Linn, 1992). However, there is evidence of multiple glutamate receptor types in the metathoracic ganglion of *Periplaneta americana* (Wafford and Sattelle, 1986), and [3H]-glutamate binding sites have been characterised in the CNS of *P. americana* (Sepulveda and Sattelle, 1989) and *Schistocerca gregaria* (Usoh *et al.*, 1989). The expression of a glutamate transporter homologue has also been demonstrated in *Drosophila melanogaster* (Besson *et al.*, 1999). Glutamate-like immunoreactivity has been observed in the Kenyon cells of the cricket *Gryllus bimaculatus* (Schürmann *et al.*, 2000), and in all parts of the locust brain (Homberg, 2002).

Glutamate has been found to be present at high concentrations in the brain of the honeybee, *Apis mellifera* (Fuchs *et al.*, 1989), compared to other classical neurotransmitters, such as dopamine, serotonin and norepinephrine. Glutamate and GABA concentrations were also found to increase and decline with age, reaching their maximum levels on day 10 of a honeybee's life. Recently, a cDNA encoding a glutamate transporter Am-EAAT was cloned from the honeybee brain (Kucharski *et al.*, 2000): the honeybee transporter is 50% identical to the human transporter subtype EAAT-2. Behavioural experiments, using the PER paradigm and olfactory conditioning in conjunction with pharmacological intervention, have also revealed a role for glutamate in the olfactory memory pathway (Maleszka *et al.*, 2000). Honeybees treated with the glutamate transporter antagonist L-*trans*-2,4-PDC show a marked impairment in the recall of long-term olfactory associative memory, while their short-term memory remains unaffected. Chapter 2 of this thesis continues the theme of using targeted pharmacological agents, and investigates the role of two NMDAR antagonists in olfactory associative memory.

1.3 Caffeine and cognition

Cognitive studies involving caffeine have largely been carried out on vertebrates, with attention focussing mainly on rats, mice, monkeys and humans. As a result, the cognitive effects of caffeine on invertebrate species, including insects, remain a mystery. The mechanism by which caffeine causes a stimulant effect in vertebrates is now beginning to be understood: caffeine blocks adenosine A_{2A} receptors in the brain (Fredholm *et al.*, 1999), and inactivates certain enzymes,

such as protein kinase A and protein phosphatase 2A (Lindskog *et al.*, 2002). Coffee has recently been found to be associated with a reduced risk of Parkinson disease (Ross *et al.*, 2000), and caffeine is able to counteract the effect of the drug MPTP, injected into the substantia nigra of rats (Gevaerd *et al.*, 2001), in order to induce a Parkinson disease type of amnesia. As a result, A_{2A}-receptor blockers are being developed as potential treatments for this condition (Chen *et al.*, 1995).

1.3.1 Caffeine and arousal

Although a vast amount of research has been carried out on the effects of habitual caffeine consumption on human cognition, there is a surprising inconsistency in the literature over how caffeine alters our mental capabilities. In general, caffeine consumption can be shown to increase the alertness and vigilance of individuals, especially in situations where arousal is low (Smith *et al.*, 1999; Beaumont *et al.*, 2001; Brice and Smith, 2001; Mikalsen *et al.*, 2001; Lieberman *et al.*, 2002; Yeomans *et al.*, 2002; Gruber and Block, 2003; Rogers *et al.*, 2003). The arousal effect of caffeine extends also to invertebrates, with caffeine-treated *Drosophila* resting less than control flies in a dose-dependent fashion (Shaw *et al.*, 2000).

1.3.2 Caffeine and memory

The effects of caffeine on working memory, STM and LTM are less clear-cut than that on arousal, and seem to depend on the time of drug administration (pre-training, post-training or pre-test) and the testing paradigm employed. Higher levels of coffee consumption have, for instance, been correlated with improved

performance in reaction time, verbal memory and visuospatial reasoning in humans (Jarvis, 1993; Hameleers *et al.*, 2000), while a slow-release dose of caffeine has been found to have a positive action on a mathematical processing task involving both LTM and STM (Beaumont *et al.*, 2001). Caffeine has also been shown to counteract the normal decline in memory performance that occurs during the course of a day in older adults (Ryan *et al.*, 2002), and lead to better recall in older women with higher levels of lifetime caffeine consumption (Johnson-Kozlow, 2002). Caffeine, when administered immediately after training in mice, facilitates the retention of an inhibitory avoidance task (Kopf *et al.*, 1999), and in a dose-dependant manner improves performance in repeated acquisition tasks, which assesses motor learning and STM. Finally, as mentioned earlier, caffeine can partly improve the acquisition of a two-way active avoidance task in mice, whose brains have been lesioned pharmacologically, to mimic Parkinson disease (Gevaerd *et al.*, 2001).

In contrast, Herz (1999) found no effect of psychoactive doses of caffeine on long-term verbal memory in humans, while neither Hudzik and Wenger (1993) nor Buffalo *et al.* (1993) were able to elicit any improvement in the delayed matching-to-sample performance of squirrel and rhesus monkeys respectively. Such inconsistencies can probably be attributed to a range of other factors, such as methodological differences, personality differences, the time of day (of testing), and the consumption of other psychoactive substances, such as alcohol, tobacco or caffeine, *etc.* (Nawrot *et al.*, 2003). There has also been some indication that natural genetic variation may be largely to blame for the varying responses of individuals to pharmacological agents: the survival time of *Drosophila*

melanogaster individuals exposed to chronic ingestion of caffeine correlates not only with the sex, but also with the genetic makeup of the individual (Carrillo and Gibson, 2002). The effects of caffeine on arousal and the learning of a relatively difficult DMTS task are investigated in Chapter 5, which also contains a study on the effects of caffeine administration on various parameters of foraging and dance behaviour (see below).

1.4 The acquisition of a complex behaviour

The usefulness of honeybees in learning studies is not limited to ease with which they can be trained in simple learning tasks. Honeybees are unique among insects (and indeed, among all animals other than vertebrates) in possessing an abstract dance 'language', by means of which the locations of food sources can be relayed to nestmates. Worker honeybees have long been known to display a complex, age-related polyethism, progressing from 'cell cleaning' and 'tending brood' to 'comb building' and 'guarding', to finally becoming foragers (Winston, 1987). The age at which foraging begins varies greatly, with bees as young as 3 days-old (Winston and Punnett, 1982) and as old as 43 days-old (Sekiguchi and Sakagami, 1966) being seen to perform their first foraging trip. As a forager, the honeybee performs some of the most cognitively challenging tasks of her short life, including the learning of landmarks (Collett and Kelber, 1988) and celestial cues (Von Frisch, 1993) to navigate, the estimation of distance using image motion (Srinivasan *et al.*, 2000), and the communication of that odometric signal to her nestmates through the famous waggle dance (Esch *et al.*, 2001). It is not

well understood how young, naïve foragers acquire the complex behaviour that is the waggle dance: do bees learn to dance through exposure to the dances of older, more experienced nestmates, or is there a genetic switch that turns on at the appropriate time in the foragers' ontogeny, thereby inducing dance behaviour? Equally likely is the possibility of a combination of both these factors.

1.4.1 The waggle dance

The waggle dance is one of the best-studied behaviours in the honeybee, and perhaps among all invertebrates (Winston, 1987). It is by means of this dance that a forager honeybee, that has been able to successfully locate a profitable food source, communicates to her nestmates the location of that food source (Von Frisch, 1993). The waggle dance, however, is performed only when the food source is beyond a certain critical distance from the hive; any food sources within this critical distance are signalled by means of a round dance. The transition point between the two dance forms is different for each subspecies of *Apis mellifera*, thus giving rise to the concept of dance 'dialects' (Von Frisch, 1993, pp. 293). In order to perform a meaningful dance, the forager must first encode the path integration vector resulting from her foraging trip in her dance, by measuring her body orientation relative to environmental features available in the nest, and also translate her flight distance into the duration of waggling (Dyer, 2002) (Fig. 1). The bees observing the dance must in turn measure the orientation and duration of the waggling run, and translate these measures into a vector corresponding to the direction and distance of the food. Various aspects of the dance language have been thoroughly investigated, as have behaviours resulting from the recruitment

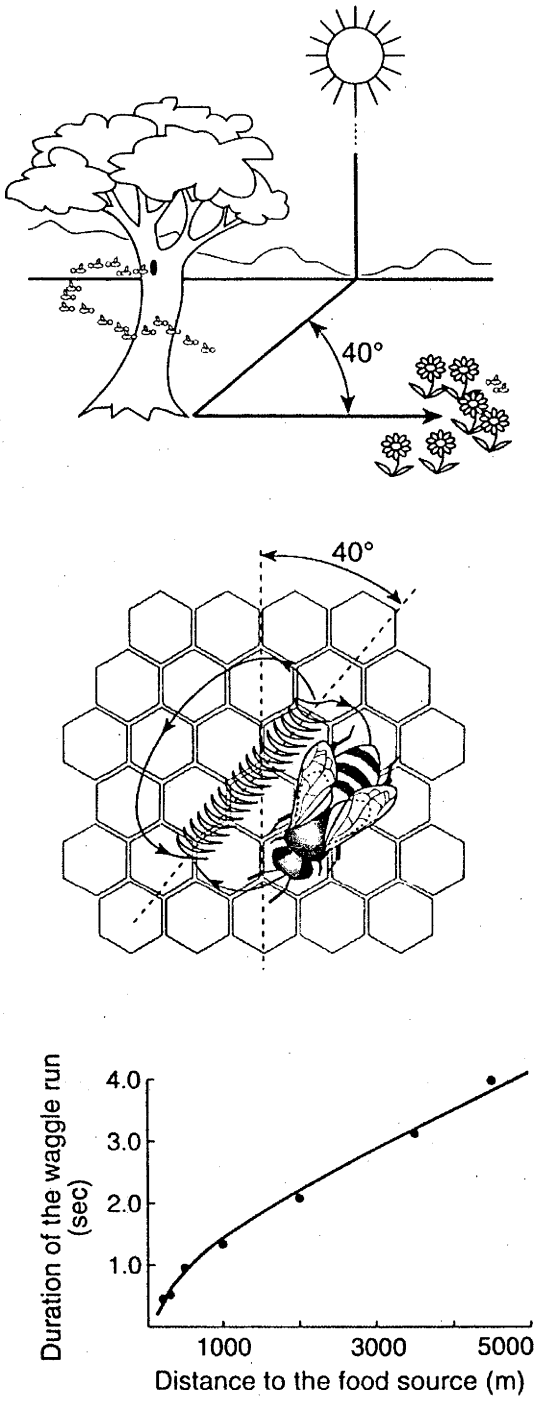


Fig. 1. The honeybee waggle dance. By means of this behaviour, the dancing forager can communicate both the direction and distance of a food source. From Seeley (1995).

of nestmates through the dance, such as the allocation of foragers to food sources, and the colony-level adjustment of the influx of nectar and pollen (Seeley, 1995). Almost nothing is known, however, about the way in which young, inexperienced workers acquire this highly symbolic and complex behaviour.

There is some evidence that the dance language has a genetic basis, at least in the context of the inheritance of dialects (Lauer and Lindauer, 1971; Rinderer and Beaman, 1995). Interesting behavioural and physiological changes have also been observed in young workers at the transition from 7 to 8 days of age, which nearly coincides with the age at which foragers perform their first orientation flights (Winston, 1987). These changes include a sizeable drop in sugar thresholds (Guez and Maleszka, unpublished), as well as an increase in the number of trials required for the habituation of the Proboscis Extension Reflex (Guez *et al.*, 2001), accompanied by the possible expression of a new nicotinic acetylcholine receptor subtype (Guez *et al.*, 2003).

However, this does not necessarily imply that the commencement of dance behaviour by adult foragers is the direct result of the turning on of a predetermined genetic switch. The dances of forager bees have been found to become slower with age, while the precision in direction and distance indicated has been found to increase with increasing foraging experience (Schweiger, 1958). Unfortunately, this study used sampling methods that confounded the dance 'tempo' (and hence the bees' motivation) with the signal indicating the distance of the food source. The topical administration of caffeine to newly-emerged worker bees has been found to reduce the age at which they can first be taught to learn an olfactory association using the PER paradigm (Maleszka and Helliwell,

unpublished data). It would therefore be interesting to see if a similar treatment also accelerates the onset of foraging and/or dancing.

1.4.2 The waggle dance in an artificial setting: tunnel experiments

It has recently come to light that honeybees estimate the distance to a food source by measuring the amount of image flow (or optic flow) that has passed over their eyes during the flight (Srinivasan *et al.*, 2000). Forager bees can therefore be tricked into thinking that they have flown large distances by artificially increasing the optic flow they experience, by, for example, training them to fly through a narrow tunnel, the walls and floor of which have been covered with a random visual texture. The proximity of the walls greatly exaggerates the perception of optic flow by the honeybees, and causes them to signal large distances in their waggle dances at the hive, in spite of their having flown only a few metres (Srinivasan *et al.*, 2000). Moreover, the dances elicited by the 'tunnel' bees are convincing enough to cause the followers of these dances to search for food at distances, which correspond to the amount of optic flow provided (Esch *et al.*, 2001).

Traditionally, such experiments have made use of a random subset of the foragers exiting the hive - as a result, the age and foraging experience of the bees is hardly ever controlled. The dance performance of the returning experimental foragers is usually pooled to generate average signal durations, with little or no attention being paid to individual differences. It also remains unclear whether the tunnel-elicited dances truly are analogous to the dances performed by bees foraging in an open, natural environment. Chapter 4 investigates the properties of

the honeybee's visual system and makes use of artificial tunnels to study the effects of specific visual patterns on the waggle dance. Chapter 5 reports an experiment where bees of known age were made to fly a distance of ~200 m in a natural environment, to investigate the effect of age and caffeine treatment on their foraging and dance behaviour.

1.5 Project aims

The aim of this thesis is to investigate various aspects of honeybee learning and memory by means of behavioural assays, pharmacological intervention and gene expression profiling. This will be achieved by means of the following studies:

a) an investigation into the role of the (vertebrate) neurotransmitter L-glutamate in the honeybee CNS using an olfactory associative learning task,

b) an investigation into the role of the adenosine A2 receptor antagonist caffeine on short term visual memory and arousal, and

c) an exploration of the effect of caffeine treatment on foraging behaviour and the possible acquisition of the symbolic dance language by juvenile honeybees.

2. Chapter 2: The role of glutamate in memory recall in the honeybee

2.1 Abstract

L-glutamate is the major excitatory neurotransmitter in the vertebrate CNS, and the NMDA receptor for glutamate is widely recognised as one that plays a vital role in learning and memory processes. In contrast to vertebrates, the involvement of glutamate and NMDA receptors in brain functions in insects is both poorly understood and controversial. Recently, however, evidence favouring a role for glutamate in learning processes in honeybee has been uncovered. The present study explores the effects of two NMDA receptor antagonists, memantine and MK-801 on learning and recall in bee of the same age, using the PER paradigm. Memantine, a medium-affinity NMDA antagonist, is well tolerated by honeybees, and injections prior to training have either no effect (in 4 and 8-day old bees), or slightly improve the performance of honeybees (7-days old) in the PER paradigm. Memory deficit was induced by injecting harnessed individuals with a glutamate transporter inhibitor, L-*trans*-2,4-PDC. This treatment impairs long-term (24hrs), but not short-term (1hr), memory in honeybees. The L-*trans*-2,4-PDC-induced amnesia is antagonised by memantine injected either before training, or before testing, suggesting that memantine restores memory recall rather than memory formation or storage. MK-801 is a high-affinity NMDA

antagonist, and has the effect of impairing the recall of LTM. It therefore appears that any disruption of glutamergic signalling pathways (either by overstimulation or complete blockage) will have a detrimental effect on the recall of long-term olfactory associative memory. These results are consistent with the distribution of glutamate-like immunoreactivity in the honeybee brain and support the role of glutamergic transmission in memory processing in this insect.

2.2 Introduction

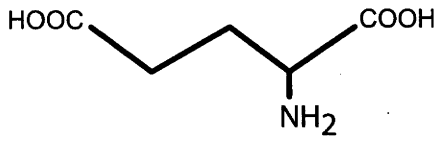
The use of easily manipulable, but behaviourally complex invertebrate model systems such as the honeybee (*Apis mellifera*) has greatly facilitated studies on learning and memory under both laboratory and natural conditions (Fahrbach and Robinson, 1995; Müller, 1996; Menzel and Giurfa, 2001). Powerful insights have been gained in the past few decades into memory dynamics, different forms of learning and conditions that optimise learning in the honeybee (Menzel, 2001). However, we are still largely ignorant of how and where memories are stored in the brain and which neurotransmitter system(s) are involved in memory consolidation and memory recall.

Acting through N-methyl-D-aspartate (NMDA) (Fig. 2.1) receptors, glutamate is integrally involved in eliciting persistent changes in synaptic function resulting in learning and memory (Milner *et al.*, 1998). By contrast, the involvement of glutamate in specific brain functions in insects and other invertebrates is both poorly understood and controversial (Kucharski *et al.*, 2000; Maleszka, 2000; Sinakevitch *et al.*, 2001) in spite of the fact that glutamate-like

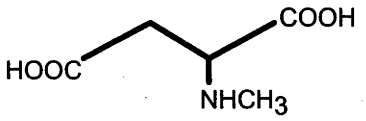
immunoreactivity has been reported in identified neuronal populations of insect brains (Bicker *et al.*, 1988; Bicker, 1999; Sinakevitch *et al.*, 2001).

Glutamate was found to impair the retention of short-term memory in *Drosophila* that had been fed the amino acid several hours prior to training in a heat avoidance task (Xia *et al.*, 1997). More recently, pre-training injections of a glutamate transporter inhibitor L-*trans*-2,4–pyrrolidine dicarboxylate (L-*trans*-2,4-PDC) (Fig. 2.1) were shown to impair long-term (24hrs) associative olfactory memory in the honeybee (Maleszka *et al.*, 2000). This result suggested a role for glutamergic transmission in memory processing in this organism and prompted the investigation into the effects of a glutamate receptor antagonist, memantine, on behaviour in the honeybee.

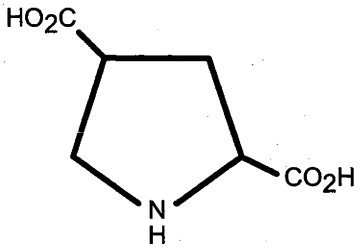
Memantine (1-amino-3, 5-dimethyl-adamantane hydrochloride) (Fig. 2.1) is a medium-affinity, uncompetitive antagonist of the NMDA receptor that shows great promise in the treatment of neurological disorders such as Alzheimer's dementia (AD) and Parkinson's disease (Parsons *et al.*, 1999; Rogawski, 2000). In comparison with high-affinity channel blocking NMDA receptor antagonists, medium-affinity uncompetitive antagonists have a reduced tendency to cause neurobehavioral side effects in laboratory animals and in humans, and consequently, are clinically well tolerated (Parsons *et al.*, 1999; Palmer and Widzowski, 2000). Memantine binds and blocks open NMDA channels more rapidly than closed channels. This 'use-dependence' property is considered as particularly desirable in enhancing the utility of this class of drugs since NMDA receptors would only be blocked when necessary (Parsons *et al.*, 1999; Rogawski, 2000). Recent clinical studies have found that memantine reduces clinical



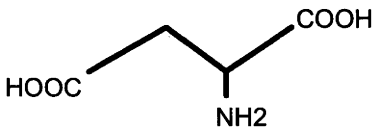
L-glutamate



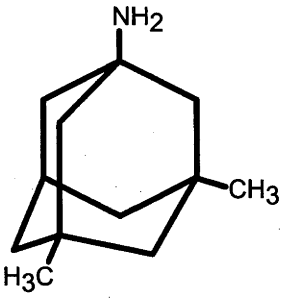
NMDA



L-*trans*-PDC



L-aspartate



Memantine

Fig. 2.1. L-glutamate and its analogues

deterioration (Reisberg *et al.*, 2003), and enhances autonomy (Rive *et al.*, 2004) in patients suffering from moderate to severe AD. Memantine hydrochloride was therefore approved as a therapeutic agent for patients with moderate to severe AD in EU countries in 2002 and USA in 2003 (Hirouchi, 2004).

MK-801 or dizocilpine is a potent, high-affinity, non-competitive NMDA receptor antagonist, that was first developed as a potential treatment for AD, but later found to have serious side effects (Farlow, 2004). Nevertheless, the efficacy and specificity of MK-801 have made it the agent of choice for investigating the effects of NMDA receptors in learning and memory (see Riedel *et al.*, 2003 for review). While MK-801 tends to impair memory formation in vertebrates, it has also been shown to eliminate the stimulatory effect of NMDA on cockroach juvenile hormone biosynthesis (Chiang *et al.*, 2002). The effect of this drug on learning and memory, however, remains unclear.

The results of the present study show that treatment with memantine alleviates memory impairment induced in honeybees by injections with L-*trans*-2,4-PDC. Memantine reverses this experimentally induced amnesia regardless of whether it is injected before training, together with L-*trans*-2,4-PDC, or injected alone before testing. The administration of these drugs at various intervals in the training protocol revealed that both act on the recall, rather than the acquisition or the consolidation of LTM. In addition, MK-801 blocks memory recall in much the same way as L-*trans*-2,4-PDC, in spite of their differing modes of action. The effects of all three drugs suggest that glutamate and NMDA receptors are involved in memory retrieval in the honeybee. These data represent the first step in

unravelling the involvement of the glutamergic system in defined brain functions in the honeybee.

2.3 Materials and Methods

2.3.1 Organism

Individual frames of brood comb were removed from an experimental hive and placed in an incubator maintained at a constant 32°C. Newly emerged bees (Fig. 2.2 a) were collected from these frames everyday, thus ensuring that the experiments were carried out only on bees of known ages.

2.3.2 Training and drug administration

The training protocol employed by Bitterman *et al.* (1983) was adopted for the present study. To facilitate handling during training and the administration of pharmacological agents, individual bees were first anaesthetised on ice, and then secured in thin-walled aluminium tubes (7 mm in diameter) using thin strips of fabric-reinforced tape (GAFFA). The bees were mounted in these tubes so as to leave the head and antennae free to move, while also leaving the dorsum of the thorax exposed (Fig. 2.2 b). Throughout the course of the experiment, any bee that seemed sluggish was discarded. Bees were fed on 1 M sucrose solution via a syringe fitted with a No. 19 needle once a day. The tubes holding the bees were then arranged in a Perspex rack and placed in an incubator overnight, to allow the bees to become accustomed to their new conditions. All bees were six days old

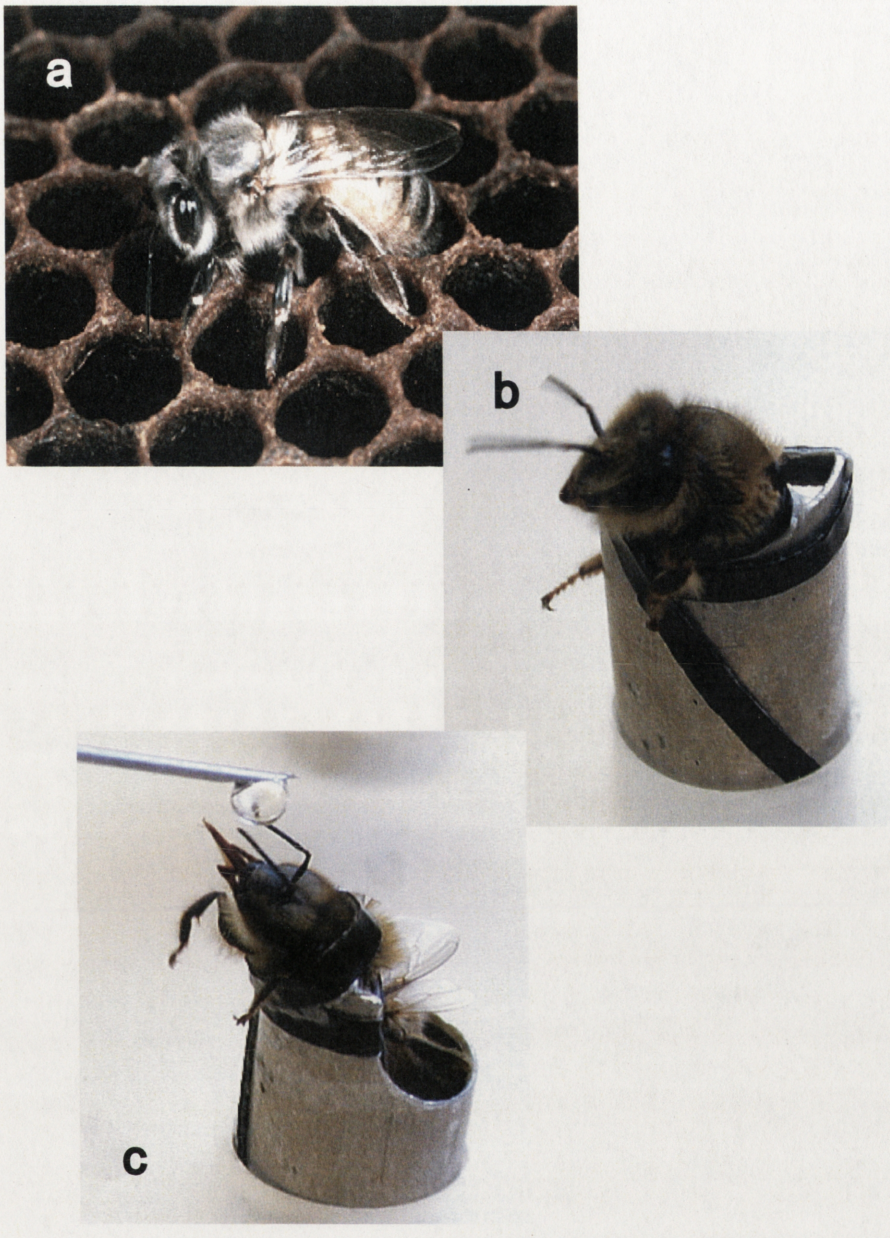


Fig. 2.2. a) A newly-emerged worker honeybee on it's brood comb. b) A 7-day old worker mounted in its stainless-steel tube. c) The training procedure, where an 8-day old worker is trained to associate, for example, the odour of limonin with sugar solution.

when mounted, seven days old when trained and eight days old when tested, except when stated otherwise.

Bees were injected with the pharmacological agent(s) of interest according to the protocol employed by Maleszka *et al.* (2000). Injections were carried out one hour prior to either a training session, a test session or both, depending on the experimental condition. Injections were carried out using a 25 µl Hamilton syringe with a repeating dispenser. Typically, 1 µl of 20 mM L-*trans*-2,4-PDC (Tocris) in Bee Ringer, 10 mM of memantine (Sigma) in Bee Ringer (20 ng/100 mg of body weight), or Bee Ringer alone (controls) was introduced into the thorax. Training consisted of teaching the bees to associate odours (conditioned stimulus, CS) with a sugar reward (unconditioned stimulus, US) (Fig. 2.2 c). Natural vanilla (4 µl/ml) in saturated NaCl solution was used as the aversive stimulus, while limonene (1 µl/ml, Sigma) in 1 M sucrose solution was the rewarding stimulus. During each training session, the bee was first allowed to smell the rewarding stimulus for 5 s, following which one antenna was touched with the stimulus, leading to the extension of the proboscis and the tasting of the sugar reward. This was repeated with the aversive stimulus. Each of these conditioning trials was repeated three times at six-minute intervals. A small exhaust fan positioned behind the bees was constantly employed throughout the duration of the experiment, in order to remove any lingering odours from the stimuli. The test for the long-term retention of associative memory (LTM) was carried out the next day, by presenting first the aversive and then the rewarding stimulus to the bees, and noting the presence or absence of proboscis extension. Bees not responding to either stimulus were discarded from subsequent analyses.

Bees responding to the aversive stimulus or to both stimuli were considered to have responded incorrectly. Short-term memory (STM) was tested similarly, the only difference being that the test was carried out one hour following a training session.

2.4 Results

Figure 2.3 shows the results of the first experiment that was designed to test the effectiveness and possible side effects of memantine injected into the thorax of honeybees of different ages, namely 4-, 7- and 8-days old. These age groups represent very young individuals (4-day old) that typically perform very poorly in the PER paradigm under standard conditions and older bees (7-8 days old) that perform significantly better under the same conditions. 7 and 8-day old bees were chosen following the finding that major changes in the honeybee cholinergic system occur at the beginning of the second week of its life (Guez *et al.*, 2001). It was reasoned that similar shifts might occur in other neurotransmitter systems. As illustrated in Fig. 2.3, 10 mM memantine (20 ng/100 mg of body weight) before training does not impair the PER conditioning in any of the tested age groups. In fact, a small but statistically significant improvement is seen in 7-day old bees following the administration of memantine. Memantine was also administered to PER conditioned bees an hour before testing, and found to have no effect on their LTM (Fig. 2.4).

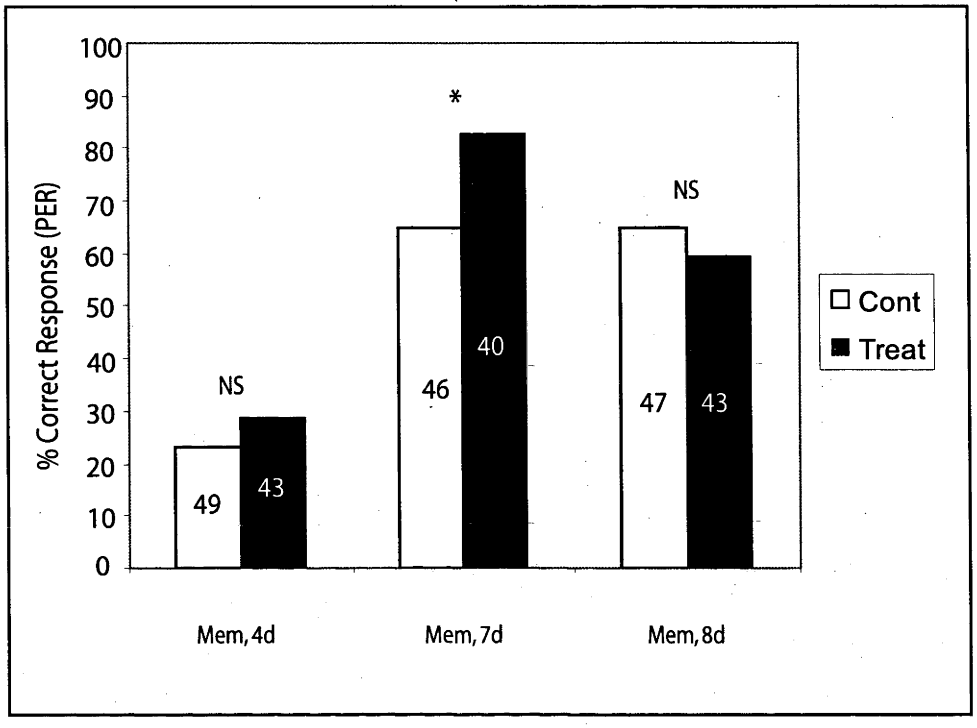


Fig. 2.3. PER learning in bees of various ages treated with 1 µl of 10 mM memantine (20 ng/100 mg body weight) prior to training. The labels under the y-axis indicate treatment conditions. The control in all cases was Bee Ringer. The numbers on the bars give the number of bees tested in each condition. * $p<0.05$; NS, no significant difference ($\chi 2$ test).

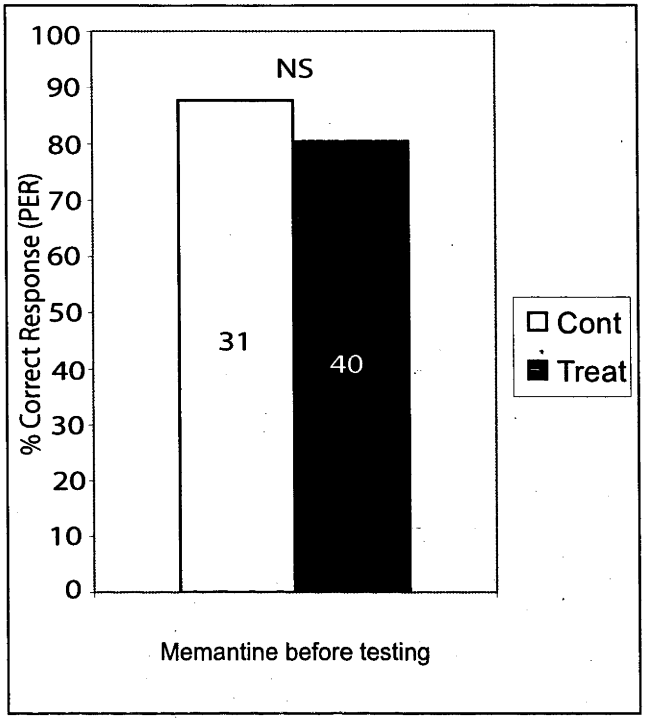


Fig. 2.4. PER learning in bees treated with 10 mM Memantine prior to testing. NS, no significant difference ($\chi 2$ test). Bees were trained when 7 days old, and tested when 8 days old. Other details as in Fig. 2.3.

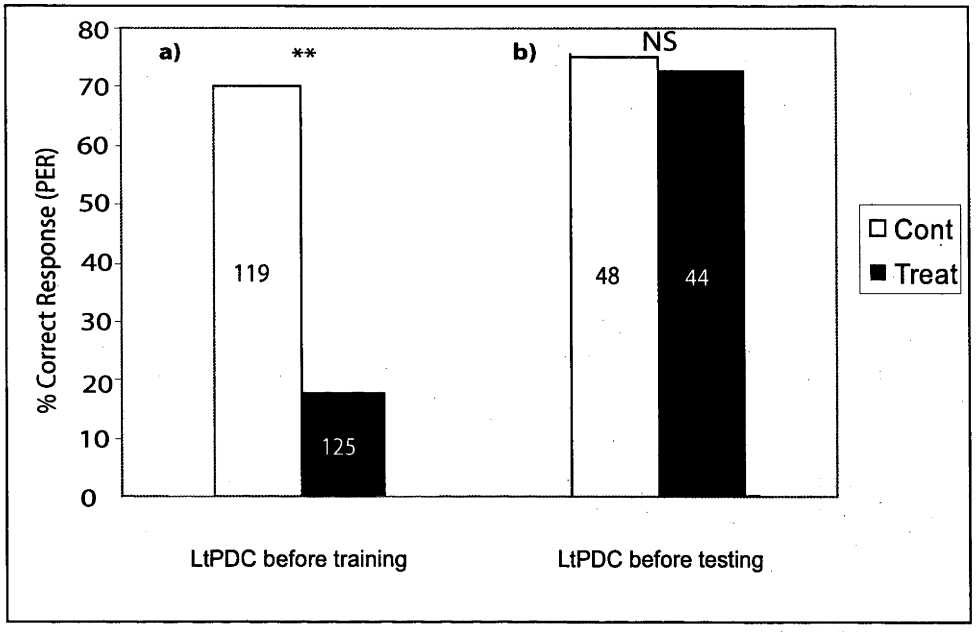


Fig. 2.5. PER learning in bees treated with 20 mM L-trans-2,4-PDC prior to training (results from Maleszka *et al.*, 2000) or to testing. ** $p<0.01$; NS, no significant difference ($\chi 2$ test). Bees were trained when 7 days old, and tested when 8 days old. Other details as in Fig. 2.3.

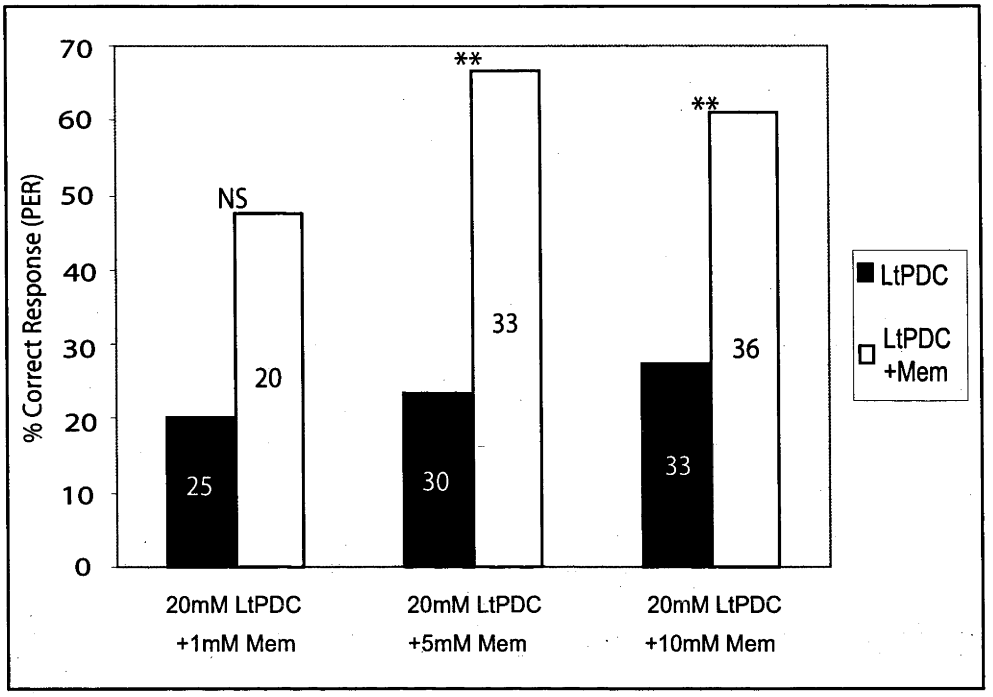


Fig. 2.6. Dependence of the level of PER learning on the concentration of memantine administered in conjunction with 20 mM L-trans-2,4-PDC prior to training. The control (dark bars) in all cases was 20 mM L-trans-2,4-PDC. Bees were trained when 7 days old, and tested when 8 days old. ** $p<0.01$ or NS, no significant difference ($\chi 2$ test). Other details as in Fig. 2.3.

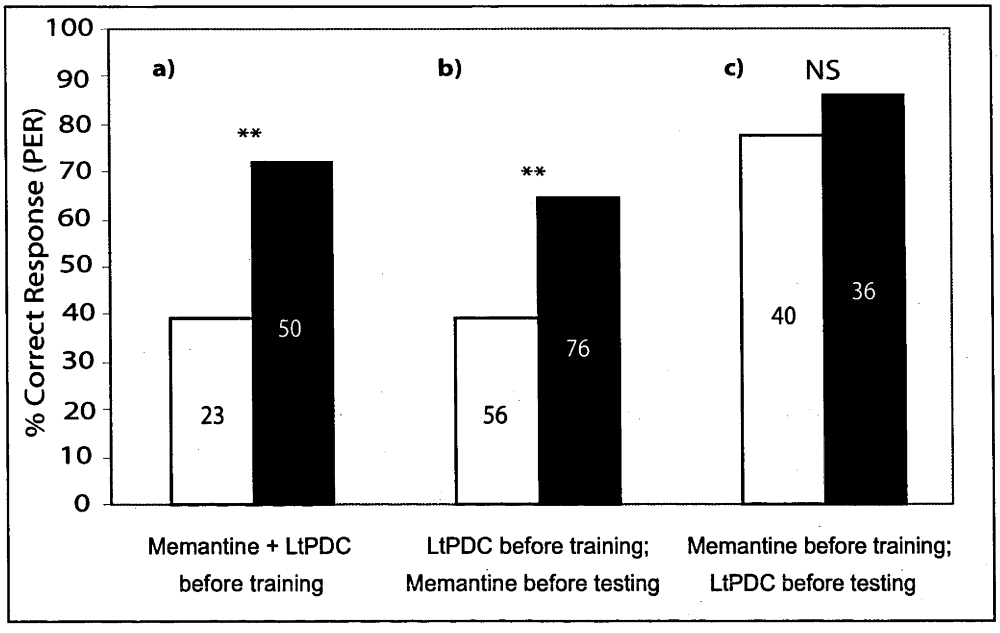


Fig. 2.7. PER learning in bees treated with 20 mM L-trans-2,4-PDC and 10 mM memantine in various combinations, or alone. Control (white bars) for experiment a), 20 mM L-trans-2,4-PDC before training; control for experiment b), 20 mM L-trans-2,4-PDC before training and Bee Ringer before testing; control for experiment c), Bee Ringer before training and 20 mM L-trans-2,4-PDC before testing. Bees were trained when 7 days old, and tested when 8 days old. ** $p<0.01$, NS, no significant difference ($\chi 2$ test). Other details as in Fig. 2.3.

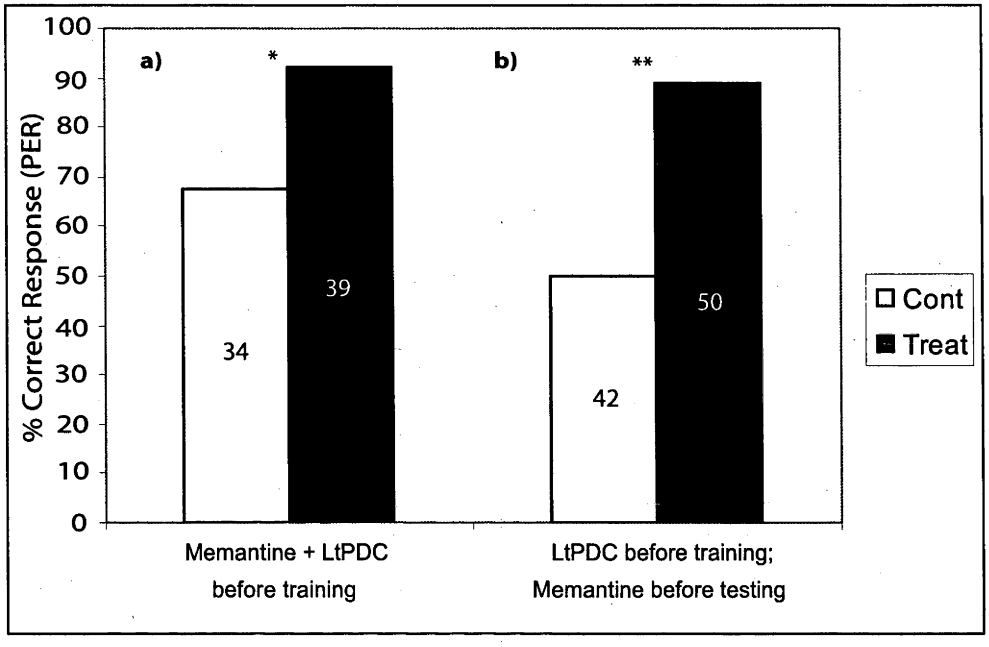


Fig. 2.8. PER learning in bees treated with 10 mM L-trans-2,4-PDC (half the concentration used in the experiments of Fig. 2.7) and 10 mM memantine in various combinations, or alone. Control (white bars) for experiment a), 10 mM L-trans-2,4-PDC before training; control for experiment b), 10 mM L-trans-2,4-PDC before training and Bee Ringer before testing. Bees were trained when 7 days old, and tested when 8 days old. * $p<0.05$; ** $p<0.01$ ($\chi 2$ test). Other details as in Fig. 2.3.

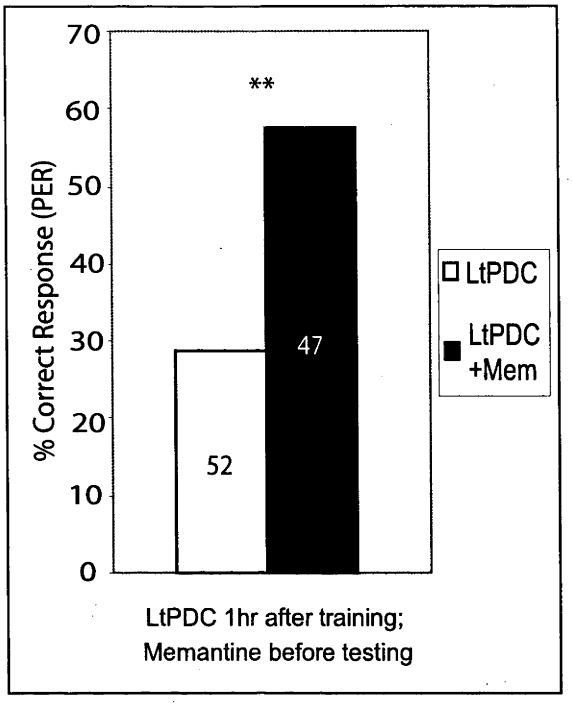


Fig. 2.9. PER learning in bees treated with 20 mM L-trans-2,4-PDC one hour after training, and 10 mM memantine one hour prior to testing. Control bees were given 20 mM L-trans-2,4-PDC after training and Bee Ringer before testing. Other details as in Fig. 2.6.

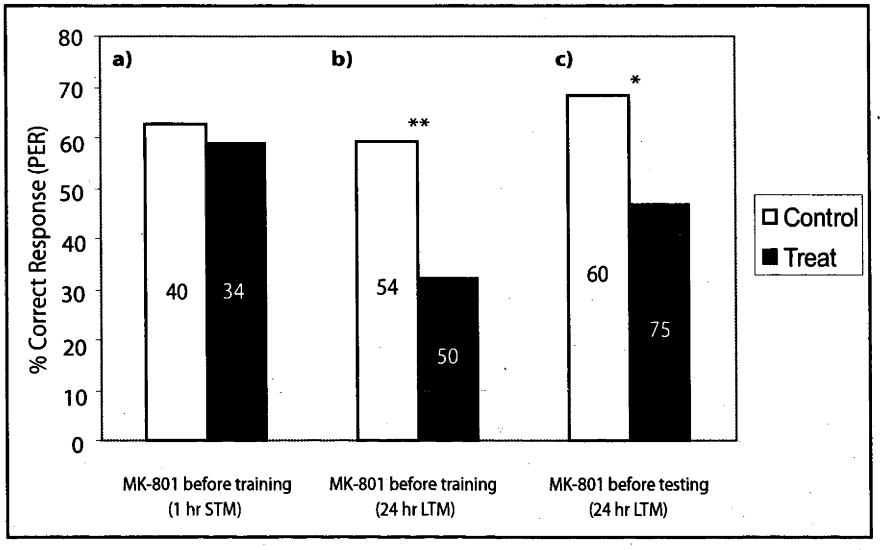


Fig. 2.10. Short-term (a) and long-term memory (b and c) of PER conditioning in bees treated with 10 mM MK-801. (a) the drug was administered before training, and bees were tested 1 h later. (b) The drug was administered before thaining, and bees were tested 24 h later. (c) The drug was administered before testing, which was carried out 24 h after the training session. Control bees in all cases were given Bee Ringer. LTM, long-term memory; STM, short-term memory. Other details as in Fig. 2.7.

Further investigations into the effect of memantine on honeybees with pharmacologically induced amnesia were carried out in a set of experiments shown in Figure 2.5. Experimental amnesia was induced by pre-training injections with 20 mM L-*trans*-2,4-PDC, a potent inhibitor of glutamate transport that causes a significant impairment of long-term (24hrs) associative memory in classically conditioned honeybees (Figure 2.5 a, Maleszka *et al.*, 2000). Injections of L-*trans*-2,4-PDC one hour before testing have no effect on memory (Fig. 2.5, b). This last result suggested either a) that L-*trans*-2,4-PDC was affecting memory formation or storage rather than recall, or b) that the kinetics of L-*trans*-2,4-PDC in the bee were such that there was a significant time delay between the administration of the drug and its effect.

Bees were then treated with 20mM L-*trans*-2,4-PDC in combination with varying concentrations (0 mM to 10 mM) of memantine. Fig. 2.6 shows that memantine acted in a dose-dependant manner, and was able to bring about a significant improvement in the performance of bees even at a dosage as low as 5 mM.

The next series of experiments was carried out to determine which step in the memory pathway (memory formation, storage or recall) was being affected by the two drugs. Once again, the administration of 10 mM memantine in conjunction with 20 mM L-*trans*-2,4-PDC prior to training restored the percentage of correct responses to normal levels (Fig. 2.7, a). An injection of 10 mM memantine one hour prior to a test was also able to bring about a dramatic improvement in the performance of bees treated with L-*trans*-2,4-PDC before training the previous day (Fig. 2.7, b). This suggested that it was memory recall

that was being acted upon by memantine. A reversal of the sequence of pharmacological intervention (memantine before training and L-*trans*-2,4-PDC before testing), however, had no effect on the responses of the animals (Fig. 2.7, c). Similar experiments were carried out with lower doses of L-*trans*-2,4-PDC, with the same outcomes (Fig. 2.8). Thus, the amnesia induced by 10 mM L-*trans*-2,4-PDC (half the earlier concentration) was also abolished by 10 mM memantine, regardless of whether if was administered simultaneously (Fig. 2.8, a) or just before testing (Fig. 2.8, b).

In order to distinguish between the two possibilities arising from the experiment reported in Fig. 2.5 (b), another group of bees was treated with L-*trans*-2,4-PDC one hour after training, followed by 10 mM memantine one hour prior to testing the following day (Fig. 5). The control bees were only treated with L-*trans*-2,4-PDC. L-*trans*-2,4-PDC was administered one hour after training in order to rule out the possibility that either memory formation or storage (consolidation) was being affected, and also to give the drug sufficient time (approximately 24 hours) to have an effect before the bees were tested. The performance of the control bees was reduced to the levels seen in previous experiments, while an injection of memantine prior to testing was able to raise it back to normal. Thus, it was the recall of LTM that was being acted upon by both drugs.

To determine if other commonly used NMDA receptor antagonists also affect olfactory memory in the honeybee, the high-affinity NMDAR antagonist MK-801 was administered to bees both before training and testing. The recall of LTM was impaired by MK-801 in much the same way as by L-*trans*-2,4-PDC

(Fig. 2.10, b and c). In addition, STM was not affected by pre-training injections of MK-801 (Fig. 2.10, a). This finding shows that MK-801 has no effect on the brain faculties needed for sensory perception, the acquisition of learning tasks or STM, but impairs the LTM of associative olfactory learning.

Finally, the effects of both antagonists MK-801 and memantine on the honeybee neuromuscular junction were evaluated. The highest concentrations tested were 20 mM (6.7 ng/bee) for MK-801 and 50 mM (10 ng/bee) for memantine. The relative mobility of bees can be easily assessed by observing mounted individuals: normal (and untreated) bees are seen to vigorously move their antennae and forelegs. No change was observed in the movement patterns of treated bees at any time during the experimental procedure, following treatment with the drugs. Judging from the relative mobility of drug-injected and control subjects, both MK-801 and memantine have no significant effects on the locomotor activities of honeybees.

2.5 Discussion

In vertebrates, much of the brain's neuronal activity is controlled by the various functional states of glutamate receptors that translate the concentration profile of neurotransmitter into a defined time course of ion flow across the postsynaptic membrane (Milner *et al.*, 1998). In insects, a growing body of evidence supports the notion that glutamate is also used for synaptic communication in the central pathways in addition to its well-established role at the neuro-muscular junction (Petersen *et al.*, 1997). Genomic sequencing has revealed highly conserved genes encoding both inotropic and metabotropic

glutamate receptors in insects (Volkner *et al.*, 2000, www.fruitfly.org) and glutamate-like immunoreactivity has been detected in insect brains, including the honeybee brain (Bicker *et al.*, 1988; Bicker, 1999; Sinakevitch *et al.*, 2001). The honeybee gene AmEAAT encoding a putative orthologue of the mammalian glutamate transporter subtype EAAT-2 is expressed in two regions of the brain, namely in the optic lobes and in a subset of Kenyon cells of the mushroom bodies, and high levels of AmEAAT message are found in pupal stages, possibly indicating a role for glutamate in the developing brain (Kucharski *et al.*, 2000). At the behavioural level, injections of a glutamate transporter inhibitor, L-*trans*-2,4-PDC, impair long-term, but not short-term, associative olfactory memory (Maleszka *et al.*, 2000). The present study shows, that a non-competitive NMDA receptor antagonist, memantine, restores L-*trans*-2,4-PDC-induced memory impairment in honeybees, regardless of whether it is injected before training or before testing. L-*trans*-2,4-PDC, too, is able to induce amnesia in bees under the same conditions, provided there is a sufficiently long delay between administration and testing. This suggests that memantine and L-*trans*-2,4-PDC restore memory recall rather than memory acquisition or storage.

In mammals, high-affinity NMDA receptor antagonists appear to have differential effects on various types of memory. Under physiological conditions, conventional inhibitors of NMDA receptors suppress long-term potentiation (LTP) and impair learning and memory (Izquierdo, 1994). On the other hand, investigations on memory functions in humans after NMDA-receptor blockade, including treatment with memantine, suggest that NMDA-receptor antagonists have differential effects on memory functions. For example, a recent study has

shown that recognition performance for objects was impaired under memantine, whereas performance on face recognition was not affected (Rammsayer, 2001). According to the current mammalian model, memantine improves cognition by ensuring a sufficient signal-to-noise ratio under conditions of increased tonic activation (noise) of NMDA receptors (Parsons *et al.* 1999). Memantine acts as a neuroprotective agent in mammals, but also can reverse NMDA-induced deficits in synaptic plasticity, both at the neuronal (LTP) and behavioural (learning) level (Parsons *et al.*, 1999). The improvement in the PER performance in 7-day old bees following memantine treatment resembles the positive symptomatological effects of this drug on learning seen in some experiments with mammals. Although the reason for this cognitive improvement is not entirely clear, some experimental data suggest that memantine can reduce the synaptic noise and in fact enhance learning, in particular in those animals that perform poorly in learning tasks (Parsons *et al.*, 1999). The honeybee performance in the PER paradigm is age-dependent, and maximum responses are typically not achieved until the age of 6-7 days (Ray and Ferneyhough, 1997; Maleszka and Helliwell, 2001). This is likely to result from a combination of factors that differentiate between younger and older bees, such as sugar thresholds, brain development and gene expression. Recent evidence has shown that a major change in the cholinergic system occurs in the honeybee brain, when they begin the second week of their lives (Guez *et al.*, 2001, 2003). Whether a similar change occurs in the glutamergic system, or whether the improvement in the PER conditioning, induced by memantine in 7-day old bees reflects an interplay of several neurotransmitter and modulatory systems, remains to be established.

The present data are so far most consistent with the idea that memantine-sensitive NMDA receptor(s) in the honeybee are involved in memory recall. It is widely accepted that learning and memory in insects is supported by paired centres called the mushroom bodies (MBs). Although it is not yet known if NMDA receptors are expressed in the MBs, these results are in good agreement with both histochemical and *in situ* hybridisation data showing that a defined area of this neuropil stains with antibodies against glutamate and with specific probes for a highly conserved glutamate transporter (Bicker 1999; Sinakevitch *et al.*, 2001; Kucharski *et al.*, 2000). Furthermore, it becomes apparent that the MBs neurons involved in memory recall and acquisition are clearly separate. This notion is reminiscent of two recent molecular studies in *Drosophila* demonstrating that synaptic output from the MBs is required for olfactory memory recall, but not for its acquisition or storage (Dubnau *et al.*, 2001; McGuire *et al.*, 2001). It is conceivable that this output in the fly is also glutamergic.

Like in other animals, memory formation in the honeybee following a 3-trial classical conditioning is a dynamic, multi-phase process that involves several brain regions and a sequence of events leading from transient interruptible memory trace to long-lasting, stable memory (Menzel, 2001; Menzel and Giurfa, 2001). The involvement of antennal lobes and octopamine in the initial stages of this process, and mushroom bodies in later stages is well established (Menzel, 2001). Other neurotransmitters, in particular acetylcholine, have also been implicated in memory processes in the honeybee (Lozano *et al.*, 2001; Shapira *et al.*, 2001). Although the cellular mechanisms underlying memory processing in the honeybee are highly conserved, it has been suggested that the temporal

dynamics of memory stages are adjusted to foraging behaviour in this insect. For example, the existence of two types of long term memory, one that is protein synthesis-independent (intervals 1-3 days) and the other that can be blocked by protein synthesis inhibitors (intervals > 3 days) may be related to flowering periods of plants in a patch (Menzel, 2001). The paradigm used in this study employed a 6-min interval between learning sessions and is expected to lead to a protein synthesis-dependent long-term memory that lasts for days (Müller, 1996). It is noteworthy that in mammals NMDA receptor-induced phosphorylation of the transcription factor CREB and expression of its target genes is an essential step in memory consolidation (Ghosh, 2002).

If glutamergic neurons in the honeybee participate in accessing of long-term olfactory memory, as the present study suggests, a major question is whether other forms of memory (e.g., spatial memory) are accessed, or encoded, by the same pathway. Clearly, more experimental data are needed to clarify this issue and to offer a comprehensive model of how information is stored in the honeybee brain and how is it accessed.

In conclusion, this study provides convincing evidence that the glutamergic system is an integral part of memory in the honeybee. The data so far are most consistent with the idea that memantine-sensitive NMDA receptor(s) in the honeybee are involved in memory recall. Given that it is very difficult to distinguish between memory formation, storage and retrieval, the experimental design used offers a convenient way to study these processes separately. Finally, since commonly used mammalian NDMA receptor antagonists have been ineffective in insects (Oleskevich, 1999), the successful usage of a medium-

affinity non-competitive antagonist, memantine, in the honeybee suggests that this drug may prove to be a valuable tool in pharmacological studies in insects.

3. Chapter 3: A comparison of aversive and rewarding stimuli in honeybee associative learning

3.1 Abstract

The role of an aversive stimulus (concentrated NaCl solution) was investigated in the context of two popular paradigms used in honeybee learning and memory studies (and elsewhere in this thesis). Free-flying bees were trained in a visual association task using a Y-maze, with either only a sugar reward associated with the correct stimulus, or with sugar and salt, associated with the incorrect stimulus. The learning curves of the two sets of bees were significantly different, and the 'punished' bees made significantly fewer repeated mistakes. Harnessed bees were also trained in an olfactory association task, using the Proboscis Extension Reflex (PER) paradigm. Three different groups of bees were trained – with one odour and a sugar reward, with one odour and a salt punishment, and with two odours, one paired with sugar and the other paired with salt. The reward-only bees achieved the highest score, the punishment-only bees achieved the lowest, and the reward and punishment bees achieved an intermediate score. The results suggest that using aversive stimuli in combination with rewarding ones allows for more reliable evaluation of learning paradigms in the honeybee.

3.2 Introduction

In recent years, honeybees have proven to be a popular model organism in studies on learning and memory at the behavioural, physiological and molecular levels (Maleszka *et al.*, 2000). Behavioural studies have shown that bees are not only capable of simple learning, *i.e.* habituation (Bicker and Hahnlein, 1994) and classical conditioning (Bitterman, *et al.*, 1983), but also more cognitively challenging tasks such as maze learning (Zhang *et al.*, 1996), and the learning of the concept of 'sameness' and 'difference' (Giurfa *et al.*, 2001). Such studies typically make use of a reward of sugar solution as either an unconditioned stimulus, with which the subject must associate the conditioned stimulus to be learnt, or simply as a means of motivating the subject to repeatedly visit the experimental apparatus. In addition, the making of an 'incorrect' choice by a subject usually results in nothing more unpleasant than the mere withholding of the reward by the experimenter in the case of tethered bees, or being forced to fly through the experimental apparatus (*e.g.* a maze) a second time to make another choice in the case of freely-flying ones. Experience with honeybee learning studies using several different experimental paradigms made it apparent that simply using rewarding stimuli could lead one to over- or underestimate the subjects' performance, depending on the paradigm being used. For instance, Y-maze studies on visual association force bees entering the base of the maze to make a choice between two different visual stimuli. Bees choosing the 'correct' stimulus are rewarded with a drink of sugar solution on entering a chamber behind the stimulus, while bees choosing the 'incorrect' stimulus merely find an empty

chamber behind the stimulus, and are released from the apparatus, to allow them to try again. It has been noticed that freely-flying bees in a Y-maze visual association task may occasionally make an incorrect choice repeatedly (10 times or more) (pers. obs.). A single such bee could, therefore, considerably reduce the score of the entire group being tested.

Studies on long-term olfactory memory based on the Proboscis Extension Reflex (PER) paradigm normally make use of a single odour-stimulus pair, which may be presented to the animal once, or in a few (three to four) trials. The animal is then usually tested approximately an hour after a single-trial conditioning regime, or after 24h, 48h or even longer, in the case of a multiple-trial conditioning regime. Testing is carried out by the presentation of only the odour (the conditioned stimulus, or CS); a bee is said to have learnt the association if she extends her proboscis in response to the CS. In such experiments, bees are often kept harnessed for over 24 hours, making it impossible to assess their nutritional status at the time of testing. It would therefore be impossible to say with certainty whether a positive PER indicated learning or hunger in a subject.

Aversive stimuli have been frequently used in classical conditioning and operant conditioning studies in a wide variety of invertebrate species, such as the sea slugs *Aplysia* (Walters *et al.*, 1981) and *Pleurobranchaea* (Mpitsos *et al.*, 1988), the locust *Schistocerca* (Forman, 1984), the fly *Drosophila* (Wustmann *et al.*, 1996), the pond snail *Lymnea* (Kojima *et al*, 1998) and the land slug *Limax* (Kimura *et al.*, 1998). Adult forager honeybees have been shown to be capable of learning to withhold the PER when presented with an odour and sugar solution coupled with an electric shock (Smith *et al.*, 1991). Very few studies, however,

(Maleszka *et al.*, 2000; Maleszka and Helliwell, 2001; Si *et al.*, 2004), have attempted to use such aversive or punishing stimuli to train bees in learning tasks, and none to my knowledge has systematically compared the performance of bees trained with sugar rewards, to those trained with aversive stimuli.

Honeybees were trained to perform association tasks in two separate paradigms. Free-flying adult foragers of varying ages were trained in a visual association task using a Y-maze, and tethered juvenile bees of known age were trained in an olfactory association task using the PER. Separate groups of bees were trained either with a reward, with an aversive stimulus, or with both. The results show that while an aversive stimulus greatly reduces the number of errors in adult bees, juvenile bees find it much more difficult to avoid a similarly aversive stimulus. The implications of such varying ability are discussed in the context of the two learning paradigms.

3.3 Methods

3.3.1 Visual association task

Individually marked bees were trained to a feeder containing sugar solution placed inside a Y-maze within the All Weather Bee Flight Facility at the Australian National University. Bees had to enter the first chamber of the Y-maze, and choose between two competing visual stimuli, namely black-and-white vertical gratings oriented at 45° and 135°. The Y-maze was constructed out of 3

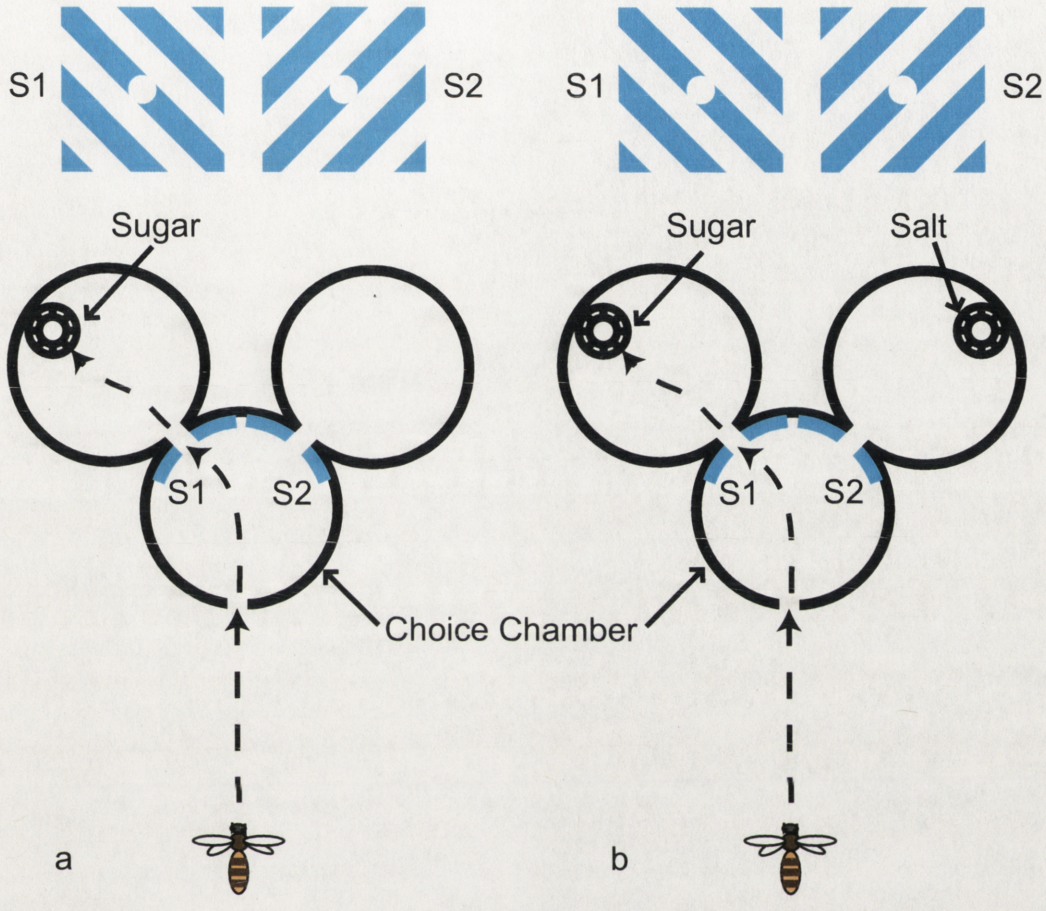


Fig. 3.1. Layout of the Y-maze experimental apparatus. a) Reward-only condition. The bee enters the choice chamber, and must choose between two competing patterns (S1 and S2). Only one (S1 in this case) leads to the chamber containing the reward. The chamber behind the incorrect pattern S2 is empty. b) Reward and punishment condition. The chamber behind the incorrect pattern S2 now holds a feeder containing concentrated salt solution.

hollow plastic cylinders, with removable, transparent Perspex lids (Fig. 3.1). Bees would have to choose the correct stimulus to enter a second chamber containing the sugar reward. Choosing the incorrect stimulus could have one of two possible outcomes: (a) some groups of bees would enter a second chamber behind the incorrect stimulus and find it empty, or (b) other groups of bees would enter a second chamber behind the incorrect stimulus, and find a feeder containing a concentrated solution of NaCl. Bees trained with condition (a) were called the 'reward-only' bees, as they were exposed only to a sugar reward, while bees trained with condition (b) were called the 'reward and punishment' bees, as they were exposed to both a sugar reward as well as a strong salt solution, which they would have found unpleasant. Bees were released from the Y-maze after each visit by removing the lid of the reward or punishment chambers. All choices of each bee were recorded, and the position of the feeder(s) was periodically switched between the two arms of the maze to prevent the development of any side-preference. Two experiments, each of 100 minutes duration, were carried out for each experimental condition. A minimum of 10 naïve bees was trained in each experiment.

3.3.2 Olfactory association task

3.3.2.1 Organism

Individual frames of brood comb were removed from an experimental hive, and transferred to an incubator, kept at a constant 32°C. Bees were collected as required on their day of emergence, and kept separately in mesh cages, until they reached the desired age.

3.3.2.2 Training protocol

The training protocol employed by Bitterman *et al.* (1983) was adopted for the present study, with some important modifications. Firstly, all subjects trained and tested in the present trials were of the same, known age, *i.e.*, seven days old when trained, and eight days old when tested. Testing was carried out 24 hours after the training procedure, thereby allowing for the quantification of long-term memory. Finally, two unconditioned stimuli (US) were used in some trials, one being associated with a rewarding conditioned stimulus (CS), and the other with an aversive CS (Maleszka *et al.*, 2000; see Section 2.3.2 for details).

Three groups of bees were trained, namely 'reward-only', 'reward and punishment' and 'punishment-only'. The 'reward-only' bees were taught to associate the odour of limonene with a reward of sugar solution. During each training session, the bee was first allowed smell the rewarding CS for 5 s, following which one antenna was touched with the US, leading to the extension of the proboscis and the tasting of the sugar reward. This was repeated three times at 6 min intervals. The 'reward and punishment' bees were trained in a similar manner to the 'reward-only' bees, with the difference that each rewarding CS-US pairing was quickly followed by the aversive CS-US pairing of vanilla and salt solution. The 'punishment-only' bees were trained in the same way as the 'reward-only' bees, but with aversive CS-US pairings of vanilla and salt instead. Tests were carried out the following day, by presenting the CS (*i.e.* the odour) to each 'reward-only' or 'punishment-only' bee, and noting the presence or absence of proboscis extension. 'Reward-only' bees were considered to have learnt the association, if they exhibited a Proboscis Extension Reflex (PER) on smelling

limonene. 'Punishment-only' bees, on the other hand, needed to suppress their PER on smelling vanilla (and without having tasted the salt during the test), to be scored as learners. 'Reward and punishment' bees were tested by presenting first the punishing and then the rewarding stimulus to each bee. The order of CS presentation was deliberately reversed in relation to the training procedure, to rule out the possibility that bees may have simply learnt the order of stimulus presentation, and not the associations themselves. These bees were considered to have learnt their associations only if they suppressed PER for vanilla, and exhibited PER for limonene. Bees exhibiting PER for the punishing stimulus or to both stimuli were considered to have responded incorrectly. A small proportion of bees (10-15%) not responding to either stimulus, and then not extending the proboscis when stimulated with sucrose, was discarded from subsequent analyses, because it was impossible to determine their learning status.

3.4 Results

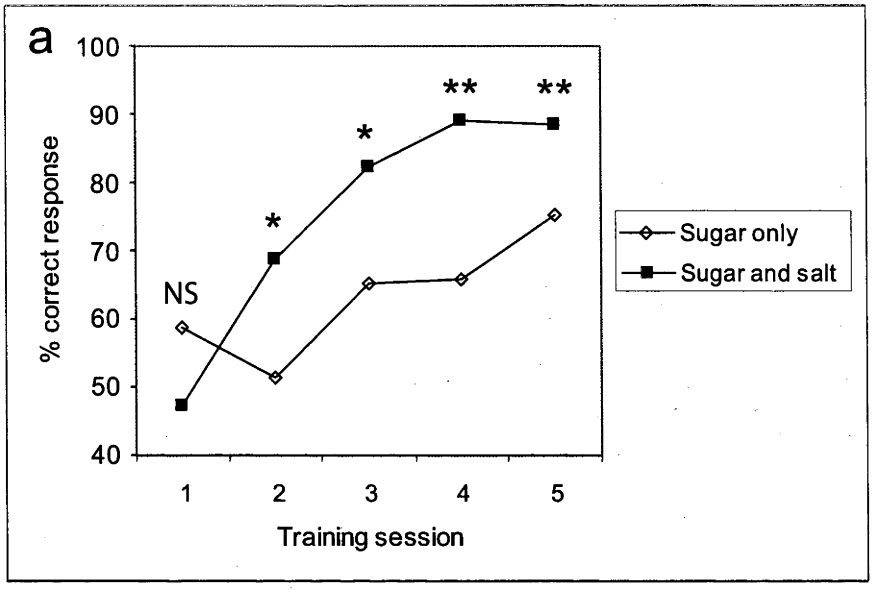
3.4.1 Visual association task

In the first experiment, bees were trained to either simply associate a visual stimulus with a sugar reward (while the competing stimulus went unrewarded), or to associate a visual stimulus with a sugar reward, while the competing stimulus was associated with a punishing stimulus of saturated NaCl solution. The punished bees were found to display a significantly steeper learning curve than their reward-only counterparts, and their final performance after five training sessions was significantly better ($p<0.01$, χ^2 Test) (Fig. 3.2 a). In fact, the

punished bees started performing significantly better from as early as training session 2, and maintained this high performance level through the remainder of the sessions. The most striking result of this experiment, however, was the difference in the frequency of repeated incorrect choices between the two groups of bees (Fig. 3.2 b). Punished bees that chose the incorrect stimulus would either leave the Y-maze and immediately choose the correct, rewarded stimulus on their next attempt, or at most make the same mistake once or twice more. This is reflected in the very low value of the mean number of repeat incorrect choices in Fig. 3.2 b. Reward-only bees also tended to correctly choose the rewarded stimulus after a single mistake, but in many cases would persist in making the same mistake as many as 3 to 10 times in a row, giving rise to a significantly higher mean number of repeat incorrect choices ($p<0.0005$, T-test).

3.4.2 Olfactory association task

The second experiment investigated the effect of rewarding and punishing unconditioned stimuli on the performance of a long-term olfactory associative task, using the Proboscis Extension Reflex (PER) paradigm. Three groups of bees were trained with reward-only, reward and punishment, or punishment-only as the unconditioned stimulus. The reward-only bees attained the highest score (77%), which was significantly greater than that of the reward and punishment bees (~70%, $p<0.005$, χ^2 Test) and the punishment-only bees (50%, $p<0.0001$, χ^2 Test) (Fig. 3.3). The reward and punishment bees attained an intermediate score, which was significantly greater than that of the punishment-only bees ($p<0.005$, χ^2 Test).



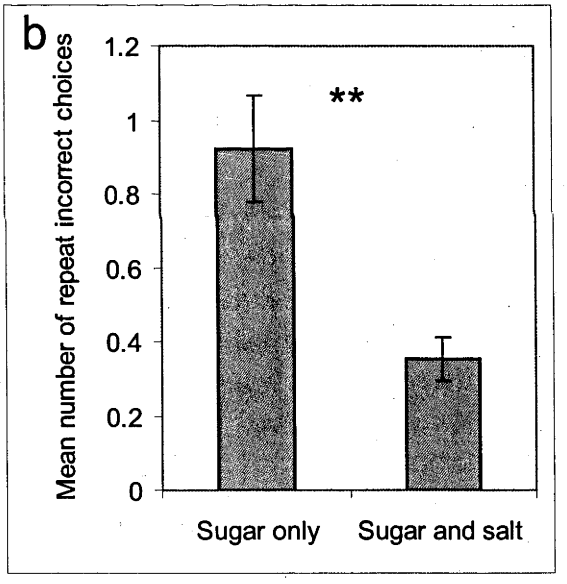


Fig. 3.2. Results of the Y-maze experiment. a) Learning curves of bees trained with either only sugar, or with sugar and salt. Each training session represents a 20 minute block. NS, non-significant; * $p<0.05$; ** $p<0.01$ ($\chi 2$ Test). b) Mean number of repeat incorrect choices for the two groups of bees. All consecutive choices made by a bee on a single visit to the Y-maze were counted. ** $p<0.0005$ (t-test).

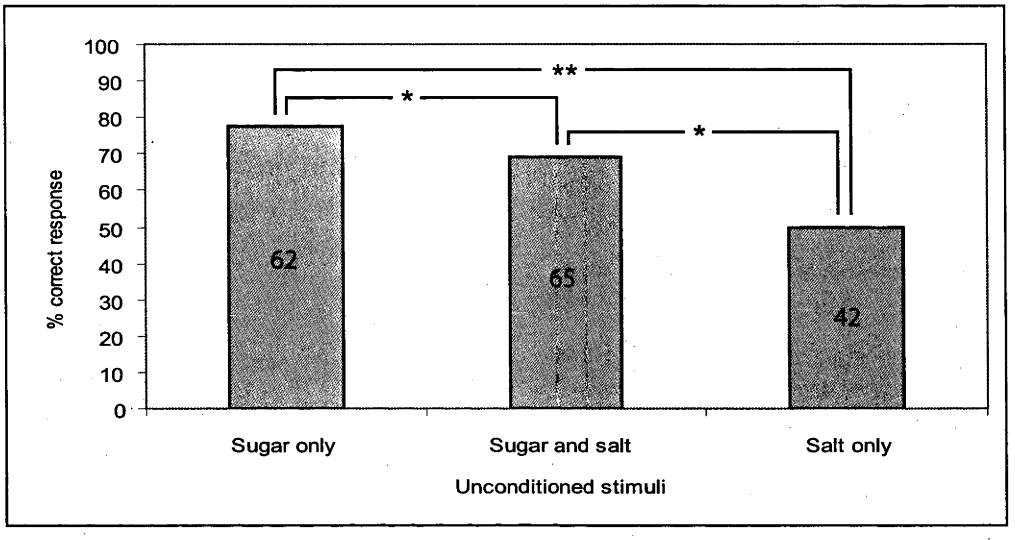


Fig. 3.3. Long-term memory of PER conditioning in bees trained with various unconditioned stimuli. The numbers on the bars give the number of bees trained and tested in each condition. * $p<0.005$, ** $p<0.0001$ ($\chi 2$ Test).

3.5 Discussion

The results of this study indicate that bees react differently to aversive stimuli in the two learning paradigms. This is to be expected, as the bees from the two experiments experienced two very different training protocols, and were expected to perform tasks of varying difficulty. For instance, the Y-maze bees were allowed to have an unlimited number of CS-US pairings, and were not expected to remember the association for more than 1-2 hours. The PER training regime differed markedly from the Y-maze one, in that the bees were only given three CS-US pairings, and were expected to remember that association over a period of 24 hours. If one also takes into account the age difference between the Y-maze and the PER bees (adult forager vs. 7 days), it becomes clear that the two experiments are not directly comparable.

Observations of the animals' behaviour during both types of experiments, however, make it clear that the salt solution used was indeed aversive: the Y-maze bees would rarely even land on the salt-containing feeder, having once assessed its contents with their antennae while still flying. Similarly, in the PER study, 'punished' bees that had successfully learnt the vanilla-salt association would vigorously move their antennae away from the source of the vanilla smell, without even having to make contact with the salt solution.

The results of the Y-maze experiment show that bees exposed to an aversive stimulus could perform the visual association task significantly better than bees that were only rewarded. The latter were also much more likely to repeatedly choose the wrong stimulus, even though this behaviour led them into a part of the maze that held no reward. The bees trained with an aversive stimulus,

on the other hand, never made the same mistake more than three times in a row, and were therefore able to learn the task faster. The reward-only bees could therefore have been learning an irrelevant cue (*e.g.* the side of the Y-maze that was last rewarded), while the presence of an aversive stimulus may have prevented the same from happening in the case of the reward-and-punishment bees.

The olfactory association experiment using the PER paradigm revealed that bees trained with only one CS-US pairing of odour and reward also achieve the highest scores. This seems to be the most common training procedure encountered in the honeybee learning and memory literature, although the subjects used in such experiments are adult foragers of unknown age (*e.g.* Fiala *et al.*, 1999; Ray and Ferneyhough, 1997; Gerber *et al.*, 1998). Nevertheless, the performance of subject bees 24 hours following three-trial olfactory conditioning seems to be consistent with the results of the present study. This training procedure, however, could cause the experimenter to confuse a true learning of the association with unrelated phenomena, such as motivation and hunger. It has been shown that the Proboscis Extension 'Reflex' is not a true reflex in the classical sense, but rather a behaviour that can be suppressed voluntarily (Smith *et al.*, 1991). It would therefore be inappropriate to score each and every bee that does not extend her proboscis as a 'non-learner'. Similarly, even though any untrained bee that simultaneously performs a PER on being presented with an odour is discarded (Bitterman, *et al.*, 1983; Fiala *et al.*, 1999; Ray and Ferneyhough, 1997; Gerber *et al.*, 1998), it would be inappropriate to score each and every bee that does extend her proboscis during testing as a 'learner'. Bees are

often left harnessed and unable to forage or feed themselves for more than 24 hours in experiments investigating long-term memory. Even though these bees are fed artificially, there would be no way of knowing their nutritional status at the time of testing. A hungry bee would probably be highly motivated to extend her proboscis in anticipation of food, regardless of whether she had learnt the required association or not. It was for this reason that the reward/punishment, double CS-US training protocol was adopted by Maleszka *et al.* (2000). Bees trained in this way have to remember to perform a PER when presented with the rewarding odour, and to withhold it when presented with the punishing one. Bees that do not respond at all during testing are discarded, if they do not perform a PER on being stimulated with sucrose solution. Bees that respond in a way other than +PER for the rewarding CS and –PER for the punishing CS can then safely be scored as 'non-learners'. Consider, as an example, bees that respond with +PER for both CSs. She may or may not have learnt the rewarding CS-sucrose association, but it can be said with confidence that she has not learnt the punishing CS-salt association. In the context of the task at hand, therefore, she is a non-learner. We believe that such a training and testing protocol provides a much more reliable measure of the learning abilities of bees.

The bees trained to conditionally suppress their PER achieved a very low score (~50%), *i.e.*, many responded to the odour of vanilla during testing, even though it was associated during training with an aversive salt solution. This is in contrast to the study by Smith *et al.* (1991), who reported a very strong suppression of about 70-80%. The latter study, however, made use of adult foragers, and investigated short-term memory after 12 paired trials. A study

similar to that of Ray and Ferneyhough (1997) therefore needs to be undertaken on the age-dependence of the conditional withholding of the PER, to determine if the difference in performance is due to age or training protocols.

On the basis of the present results, it seems advisable to use aversive stimuli in conjunction with rewarding ones, at least when making use of the PER paradigm. While aversive stimuli lead to a faster learning of the task in Y-maze experiments, they would produce much more meaningful results in PER experiments.

4. Chapter 4: Honeybee Navigation: Visual odometry in an artificial setting

4.1 Abstract

Recent work has revealed that honeybees determine distance flown by gauging the extent to which the image of the environment moves in the eye as they fly toward their destination. Artificially enhancing the amount of optic flow experienced, by making bees fly through short stretches of a narrow tunnel, appears to elicit waggle (instead of the usual round) dances, which signal distances of several hundred metres. It has been shown that certain parameters of the waggle dance change with both the age and the foraging experience of the bees in question (Schweiger, 1958). Given that bees of all ages and foraging experiences are used in 'dance' experiments, it is possible that the odometric information relayed by bees returning from tunnels is not entirely representative of either the distance travelled, or the optic flow experienced. The present study examines the properties of the bees' visually driven 'odometer' in tunnels lined with a range of visual patterns, through the analysis of the dances when the bees return to the hive. It also investigates the consequences, of using a mixed group of bees, for the distance signal relayed at the hive. This study therefore complements the following chapter (Chapter 5), where the dances of bees of known age, flying

in a natural, open environment, are investigated. The results of the present study show that the odometric signal is relatively unaffected by variations in the contrast and spatial frequency content of the patterns, and that a strong signal is generated even when the walls or the floor of the tunnel are entirely devoid of optic-flow cues. Secondly, the dances performed by 'tunnel' bees contain a significant non-waggle component, even in conditions of high optic flow. These results are discussed in the contexts of the honeybee's visual capabilities and the ontogeny of the waggle dance behaviour respectively.

4.2 Introduction

When a scout honeybee discovers an attractive patch of flowers, she performs the famous "waggle dance" for distances beyond a certain threshold value that advertises the location of the food source to her hivemates (Von Frisch, 1993). The dance consists of a figure-of-eight, interspersed by a segment in which the bee waggles her abdomen from side to side. The duration of this "waggle phase" conveys to the potential recruits the distance of the food source from the hive: the longer the duration of the waggle, the greater the distance (Von Frisch, 1993). This information is used by the recruited bees to locate the food source. Clearly, then, the scout as well as the recruits is able to gauge how far they have flown in search of food.

Early studies had concluded that bees estimate distance flown by gauging the amount of energy they expend to reach the destination (for review, see Von Frisch, 1993). However, recent work has shown that it is actually the optic flow

experienced by the eye (that is, the speed of motion of the image of the environment) that is integrated over time to obtain an estimate of distance traveled (Esch and Burns; 1995, Srinivasan *et al.*, 1996, 1997, 2000; Esch *et al.*, 2001). Foraging bees that have been made to fly through short, narrow tunnels lined with a visual texture return to the hive to signal a distance of several hundred metres; replacing the visual texture with axial stripes (that generate no optic flow) makes bees revert back to signalling short distances (Srinivasan *et al.*, 2000). If bees do indeed guage distance traveled by measuring optic flow and integrating it over time, it is pertinent to enquire into the properties of their visually driven 'odometer'. Given that the environment through which a bees flies can vary substantially in terms of its visual properties, such as contrast, texture, and the distribution of objects, it is important to know whether, and to what extent, the bee's perception of distance flown is affected by these environmental variables. Furthermore, the bees used in the experiment described above would have been of a wide and unknown range of ages. Given that the accuracy of the distance and direction being signalled varies significantly with the age of the dancer (Schweiger, 1958), what might be the distance information being relayed by these 'tunnel' bees back at the hive? The existence of any communicative intent whatsoever in the dance 'language' has been challenged in the past, partly on the basis of the large variance in both the distance and direction being signalled by foraging bees (see Gould and Gould (1988) for review). Might the differences in age and experience explain the observed noise or error in the dances? Moreover, might 'stronger' optic flow signals be relayed more faithfully by tunnel bees than 'weaker' ones?

The tunnel experiment described above (Srinivasan *et al.*, 2000) offers a convenient means of exploring this question under controlled laboratory conditions, since outdoor flights of a few hundred metres can be simulated in the laboratory by flying bees through narrow tunnels a few metres long. Thus, one can investigate the effects of varying the contrast, texture or other attributes of the environment by varying the properties of the visual patterns that line the walls and floor of the tunnel, and analysing the resulting dances. The results of the present study indicate that the visual system of the honeybee is robust to changes in the contrast and spatial frequency characteristics of the visual scene. The dances elicited are not 'pure' waggle dances, however; this phenomenon could be due to the range of ages of the bees used in the experiment.

4.3 Materials and Methods

4.3.1 Tunnel

Bees from an observation hive were trained to fly to a tunnel placed outdoors with its entrance at a distance of 35 m from the hive. The walls of the hive were made of clear Perspex sheets, which facilitated the viewing and filming of bee dances. The tunnel was was oriented with its entrance facing the hive. It was 11 cm wide, 20 cm high and 6 m long in all experiments except one in which it was 2 m, 4 m or 8 m long. A strip of black insect screen cloth formed the roof of the tunnel, allowing the observation of bees within (Fig. 4.1 a). Bees were trained to fly to a feeder containing sugar solution placed at the far end of the

tunnel, which was kept closed. Bees could therefore enter and leave the tunnel only through the near end. Up to 20 bees were marked at the feeder for each experimental condition. Dances performed by marked bees returning from the tunnel were filmed at the hive using a digital camera, and later analysed (Fig. 4.2 b).

The side-walls and floor of the tunnel were lined with various black-and-white patterns and gratings. In the first series of experiments, the walls and floor of the tunnel were lined with a checkerboard pattern of square size 3.2 cm, or with axial stripes of period 8cm. In the second series, a randomly textured Julesz pattern with a pixel size of 1cm and a pattern of axial stripes were used in various combinations. As controls, bees were made to fly to feeders placed at either 35m or 41m from the hive entrance. In a third experimental series, the side-walls and floor of the tunnel were lined with vertical square-wave gratings with a period of 3.6cm and contrasts ranging from 20% to 92%. In a fourth series, the patterns were sinusoidal gratings of medium contrast and varying spatial periods (values given in "Results"). In addition, the checkerboard pattern, as well the axial striped pattern was used as control patterns in the latter two series of experiments. The checkerboard pattern was used on three separate occasions during the study to provide a baseline against which to compare the data obtained from the other experimental conditions. The axial pattern was used to create a condition in which the optic flow experienced by bees flying through the tunnel was close to zero. This was because flight in the direction of the stripes produced very little or no apparent motion of the images of the walls and floor in the eyes.

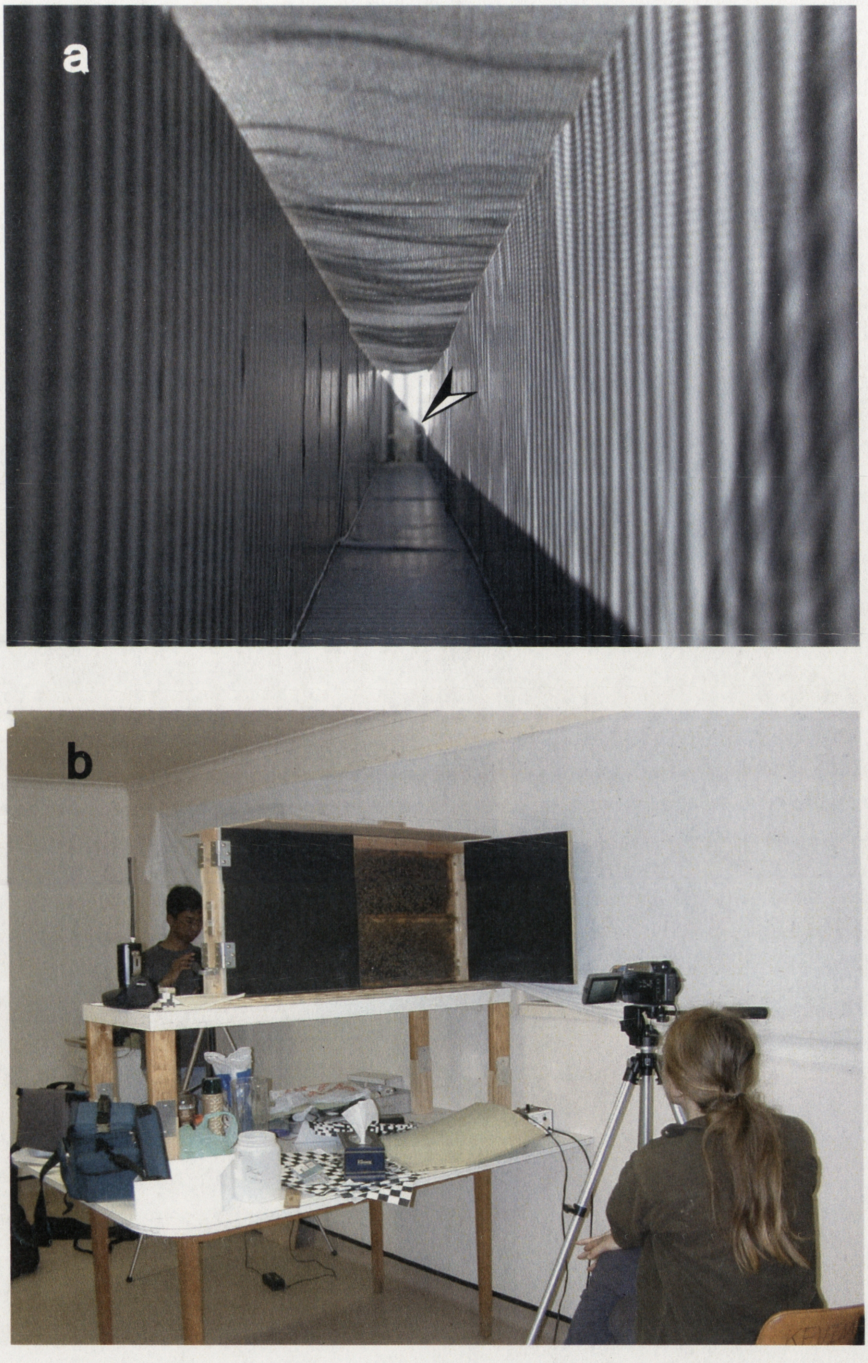


Fig. 4.1. a) a view of the interior of the experimental tunnel from the entrance, showing a sinusoidal grating on the walls and floor, and a feeder (arrowhead) at the distal end. b) The video recording of dances on the observation hive inside the beehouse.

The patterns were printed on a laser printer using a computer running a graphics program. The contrasts of the patterns were measured by using a photodiode that had a linear intensity-response function and a visual field considerably narrower than the smallest pixel or stripe width that was used.

4.3.2 Data analysis

For each experimental condition, the dances performed by the marked bees upon their return to the hive were analysed. A dance typically consisted of a number of loops, alternating between the clockwise and counterclockwise senses. Some of these loops displayed a waggle component, whereas others carried no detectable waggle component. For each dance, two parameters were measured: (i) the percentage of waggle loops; and (ii) the mean duration of the waggle component throughout the dance, assigning a waggle duration of zero to each loop that had no waggle. The data were analysed in this way because all dances - including those performed by bees returning from feeders positioned at a considerable distance within the tunnels – were found to contain a certain number of loops in which there is no waggle. In the case of the classical "waggle dance" that a bee performs after returning from a food source at a large distance (Von Frisch. 1993) the percentage of waggle loops is large. On the other hand, in the case of the classical "round dance" that a bee performs after returning from a nearby food source (Von Frisch. 1993), the percentage of waggle loops is low. It was therefore decided, that the analysis of the dances should (a) take the non-waggle loops as well as the waggle loops into account in measuring mean waggle duration and (b) use the percentage of waggle loops as an additional measure of

the bees' perception of how far they had traveled. No obvious 'sickle dances', which are meant to be performed as a transition between round and waggle dances, were observed. However, as the direction component of the dances in this set of experiments was not analysed, it is possible that some of the tunnel dances might have been of a 'sickle' type. The Fisher's Least-Significant-Difference Test was used to test for statistically significant differences between the mean waggle durations obtained for different conditions. Pseudoreplication at the level of the bee was inevitable (and considered acceptable) in this experimental design: all responses for a certain condition were pooled, including repeated dances for each bee. It would not have been feasible to train large enough numbers of bees to allow the recording of only a single dance from each bee.

4.4 Results

4.4.1 Dances of bees returning from tunnels

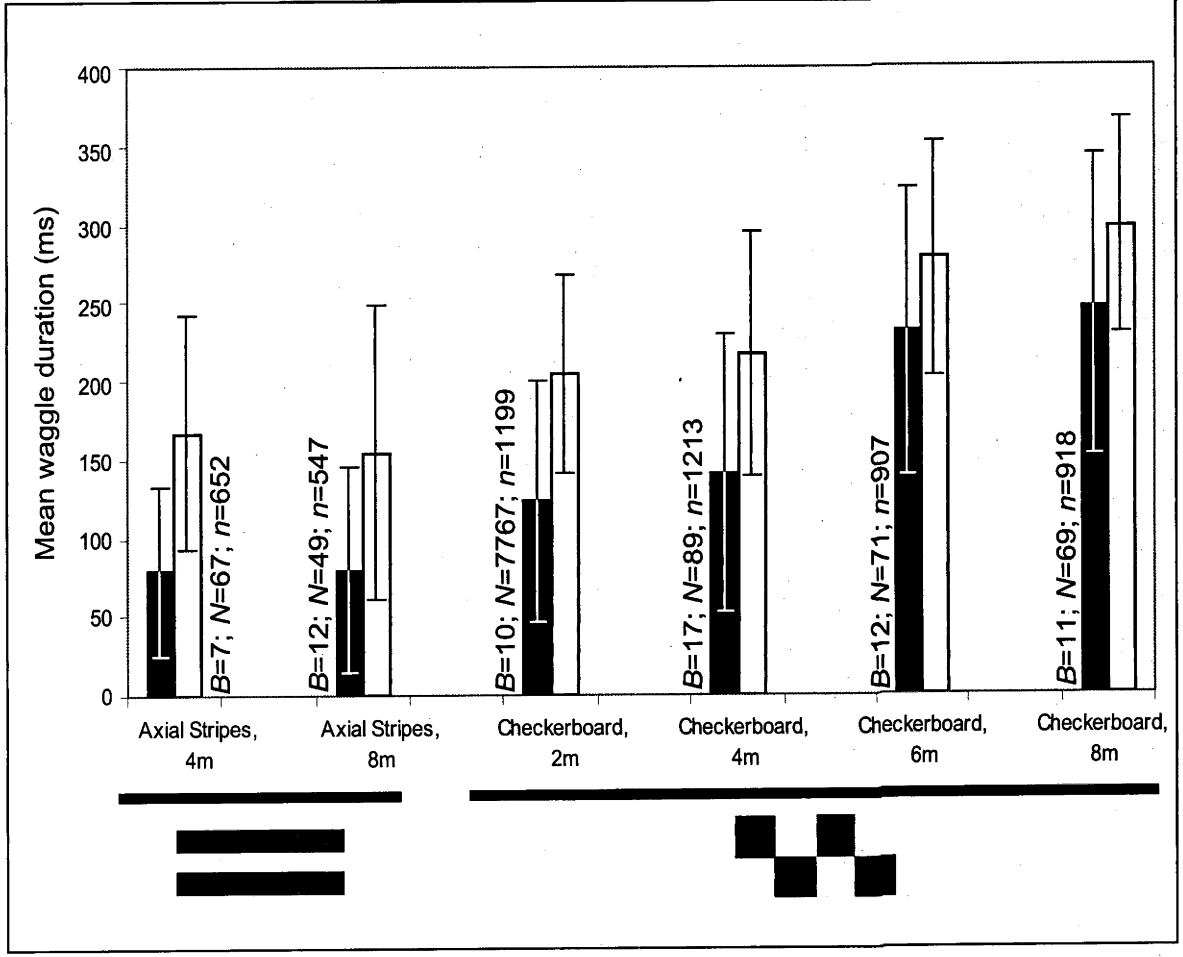
Bees were first trained to fly to a feeder placed inside a tunnel in which the walls and floor were lined with a checkerboard pattern. The dances of bees returning from the tunnel were filmed with the feeder placed at distances of 2m, 4m, 6m and 8m from the tunnel entrance. The mean waggle duration increased systematically with distance flown (Fig. 4.2), as did the percentage of waggle loops (Fig. 4.3). At a distance of 8 m into the tunnel, the mean waggle duration is ca. 250 ms.

4.4.2 Contribution of lateral and ventral visual fields to odometry

This experiment was carried out to determine which region, or regions of the eye are involved in the measurement of the optic flow that is used by bees to assess distance traveled. Also, how sensitive is the calibration of the honeybee's visual odometer to deprivation of optic flow in specific regions of the visual field?

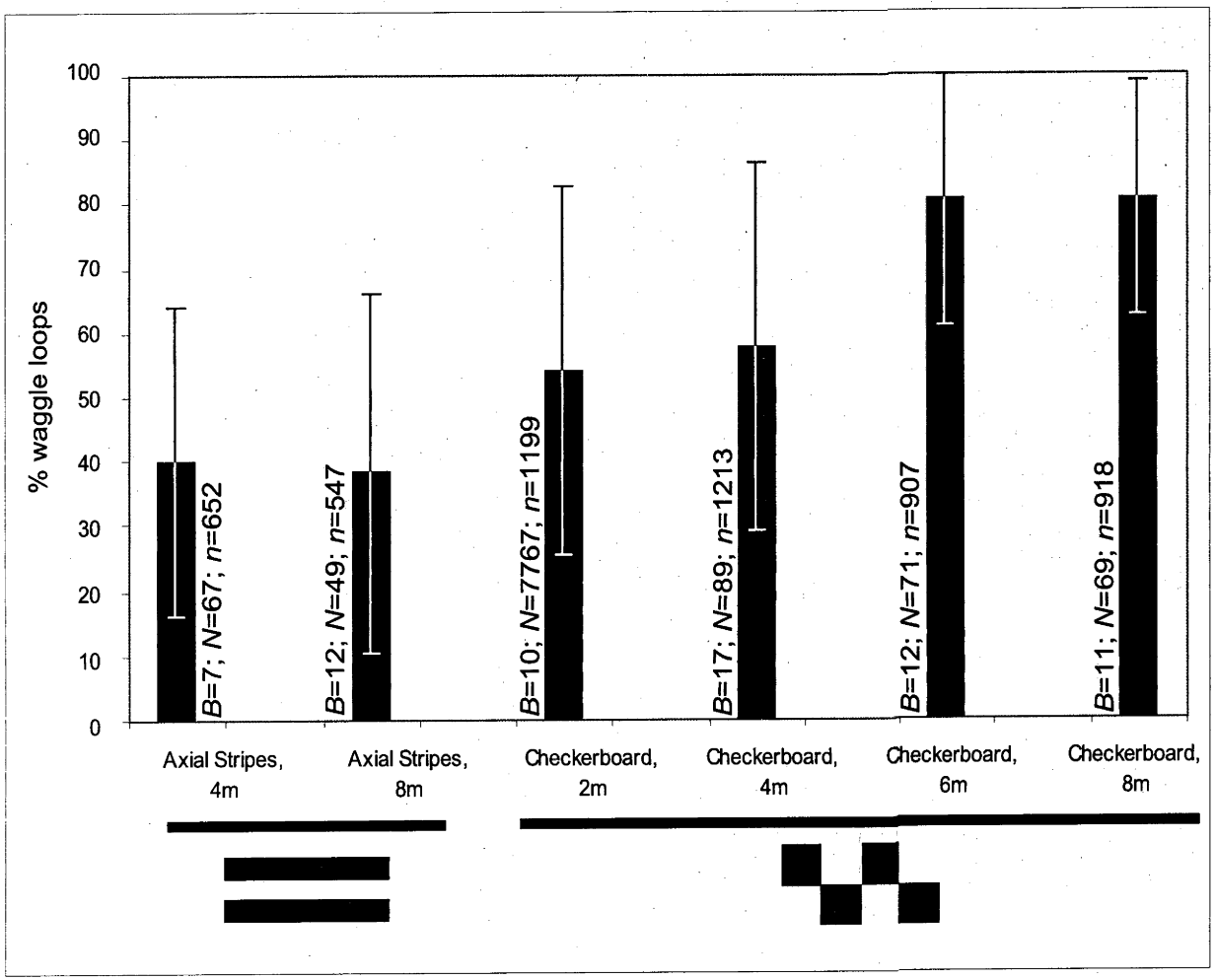
Bees were made to fly through a tunnel whose floor, or side-walls, or all surfaces were lined with a random black-and-white Julesz pattern to provide motion cues. The remaining surface(s) were lined with an axial striped pattern, and thus provided negligible optic flow cues. The mean waggle duration and percentage of round loops for dances of marked bees returning from the tunnels under these various conditions were measured, as described in "Methods".

The results revealed that when the tunnel provided optic flow on all surfaces (walls as well as floor), the mean waggle duration was ca. 210 ms (Fig. 4.4). This was the experimental condition that elicited the largest mean waggle duration (Fig. 4.4), as well as the largest percentage of waggle loops (82 %, Fig. 4.5). When the walls of the tunnel provide optic flow, but not the floor, the mean waggle duration decreased slightly (to 180 msec, Fig. 4.4). The percentage of waggle loops also displayed a slight decrease (Fig. 4.5). When the floor contributed optic flow, but not the walls, the mean waggle duration decreased by a further, small amount (Fig. 4.4). This was mirrored by a further, small decrease in the percentage of waggle loops (Fig. 4.5). Comparing the data in the second and third columns of Figs. 4.4 and 4.5, we see that the walls of the tunnel make a slightly, but significantly greater contribution to the odometric signal than does the floor. The striking feature of the dances that were elicited by these three



	Ax,4m	Ax,8m	Ch,2m	Ch,4m	Ch,6m	Ch,8m
Ax,4m						
Ax,8m	N.S.					
Ch,2m	**	**				
Ch,4m	**	**	N.S.			
Ch,6m	**	**	**	**		
Ch,8m	**	**	**	**	N.S.	

Fig. 4.2. The effect of varying tunnel length on the mean waggle duration (black bars) and the mean pure waggle duration (white bars) of bees returning to the hive after being rewarded inside the tunnel. Values are means ± S.D. for each experimental condition; in this and all figures, B denotes the number of bees, N the number of dances analysed, and n the total number of loops (waggle + non-waggle) analysed. The walls and floor of the tunnel (length 2, 4, 6, or 8m) were lined with a checkerboard pattern in all but two cases, where axial stripes were used instead. The table to the right of the histogram shows the results of a pairwise Fisher's Least Significant Difference Test for differences in the values of mean waggle duration; * $p<0.05$; ** $p<0.01$; NS, no significant difference ($p>0.05$).



	Ax,4m	Ax,8m	Ch,2m	Ch,4m	Ch,6m	Ch,8m
Ax,4m						
Ax,8m	N.S.					
Ch,2m	**	**				
Ch,4m	**	**	N.S.			
Ch,6m	**	**	**	**		
Ch,8m	**	**	**	**	N.S.	

Fig. 4.3. The effect of varying tunnel length on the proportion of waggle loops. Other details as in Fig. 4.2.

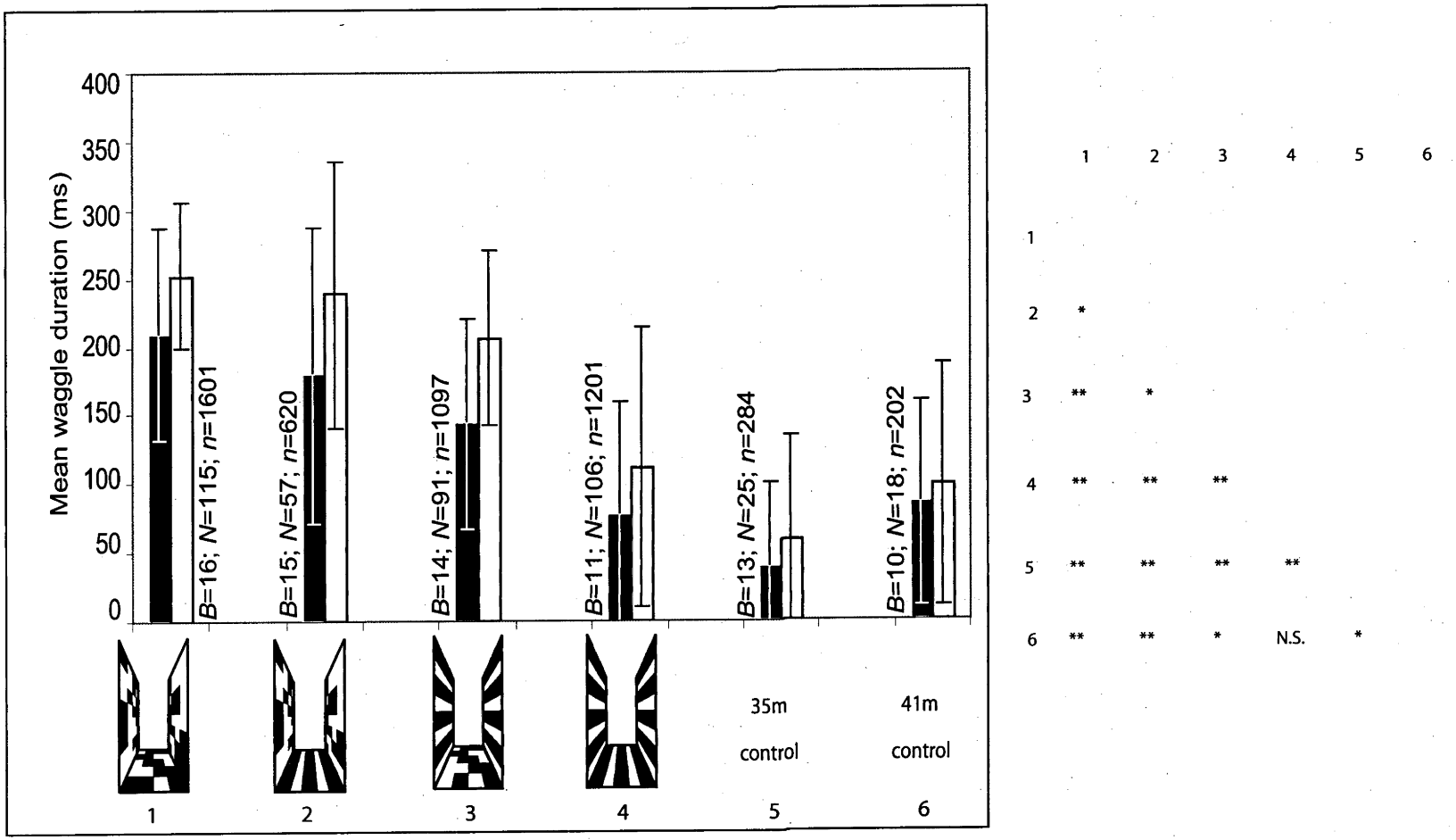
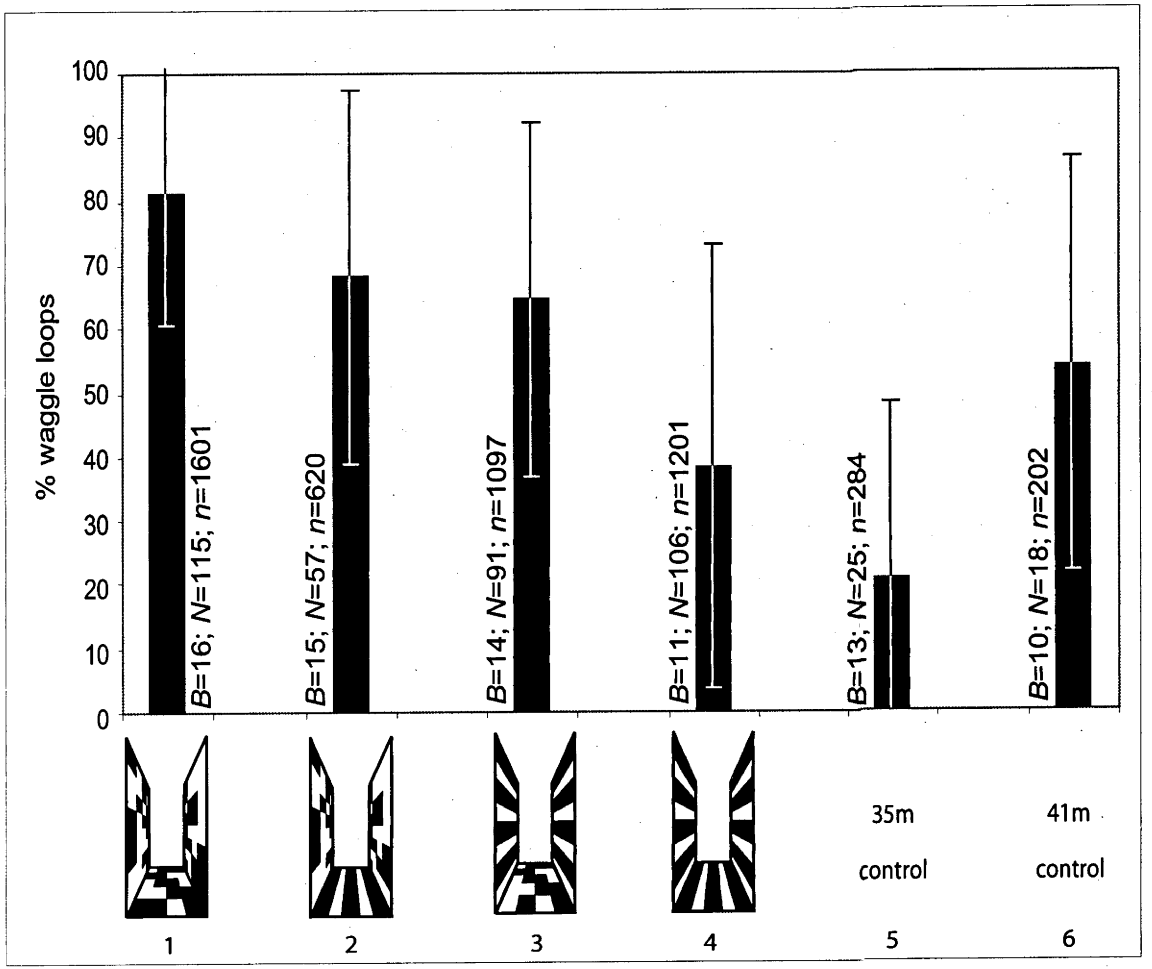
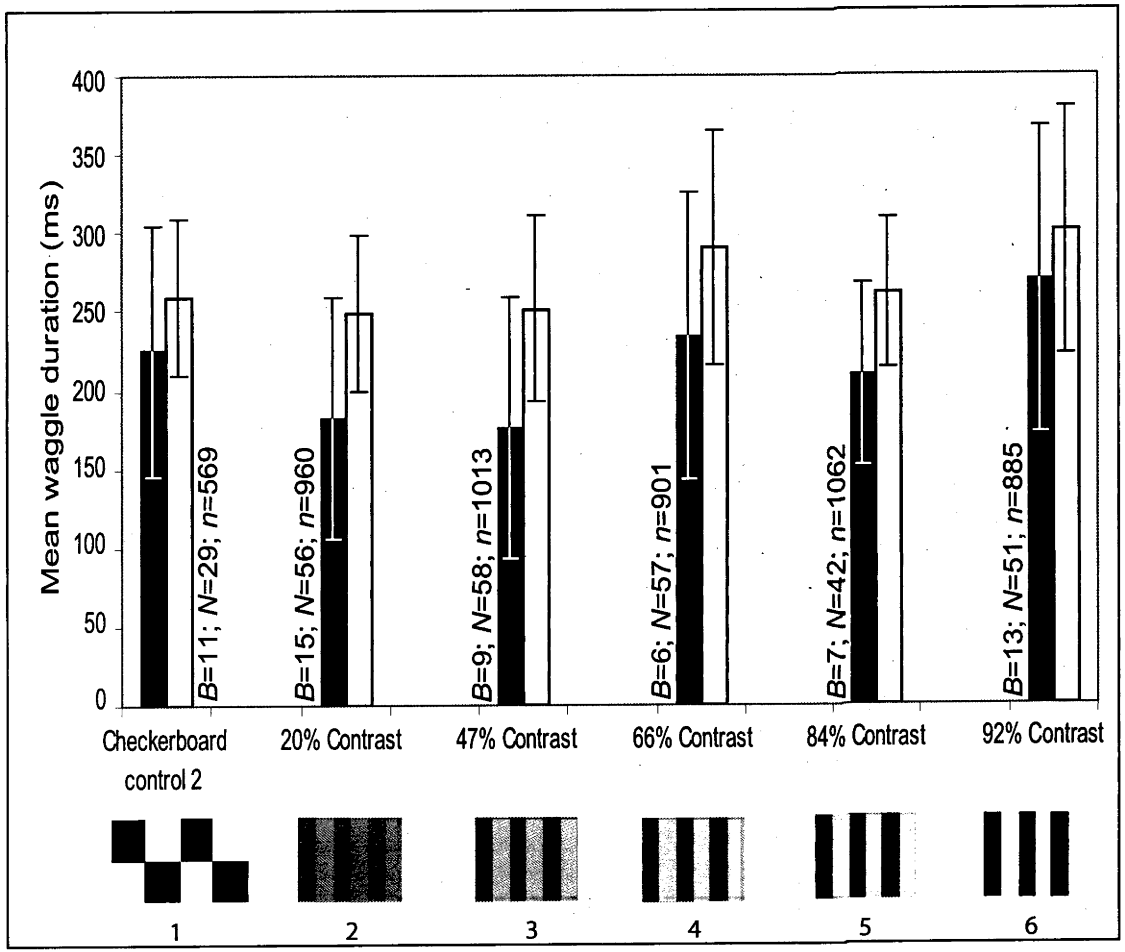


Fig. 4.4. The contribution of the ventral and lateral fields of view to odometry. Variation in mean waggle duration (black bars) and the mean pure waggle duration (white bars) of bees after flying in a tunnel with different surfaces providing optic flow (random Julesz pattern) or no optic flow (axial stripes) (indicated below). Controls, behaviour after outdoor flight to tunnel entrance; see text for details. Other details as in Fig. 4.2.



	1	2	3	4	5
1					
2	*				
3	**	N.S.			
4	**	**	**		
5	**	**	**	**	
6	**	**	*	N.S.	*

Fig. 4.5. The contribution of the lateral and ventral visual fields to odometry. The histogram shows the variation of the proportion of waggle loops. Other details as in Fig. 4.4.



	1	2	3	4	5
1					
2	*				
3	**	N.S.			
4	N.S.	**	**		
5	N.S.	*	*	N.S.	
6	*	**	**	*	**

Fig. 4.6. The effect of contrast on mean waggle duration (black bars) and the mean pure waggle duration (white bars). The walls and floor of the tunnel were lined with square-wave gratings of various % contrasts, as shown. A 92% contrast checkerboard pattern was used as a control to provide a baseline value. Other details as in Fig. 4.2.

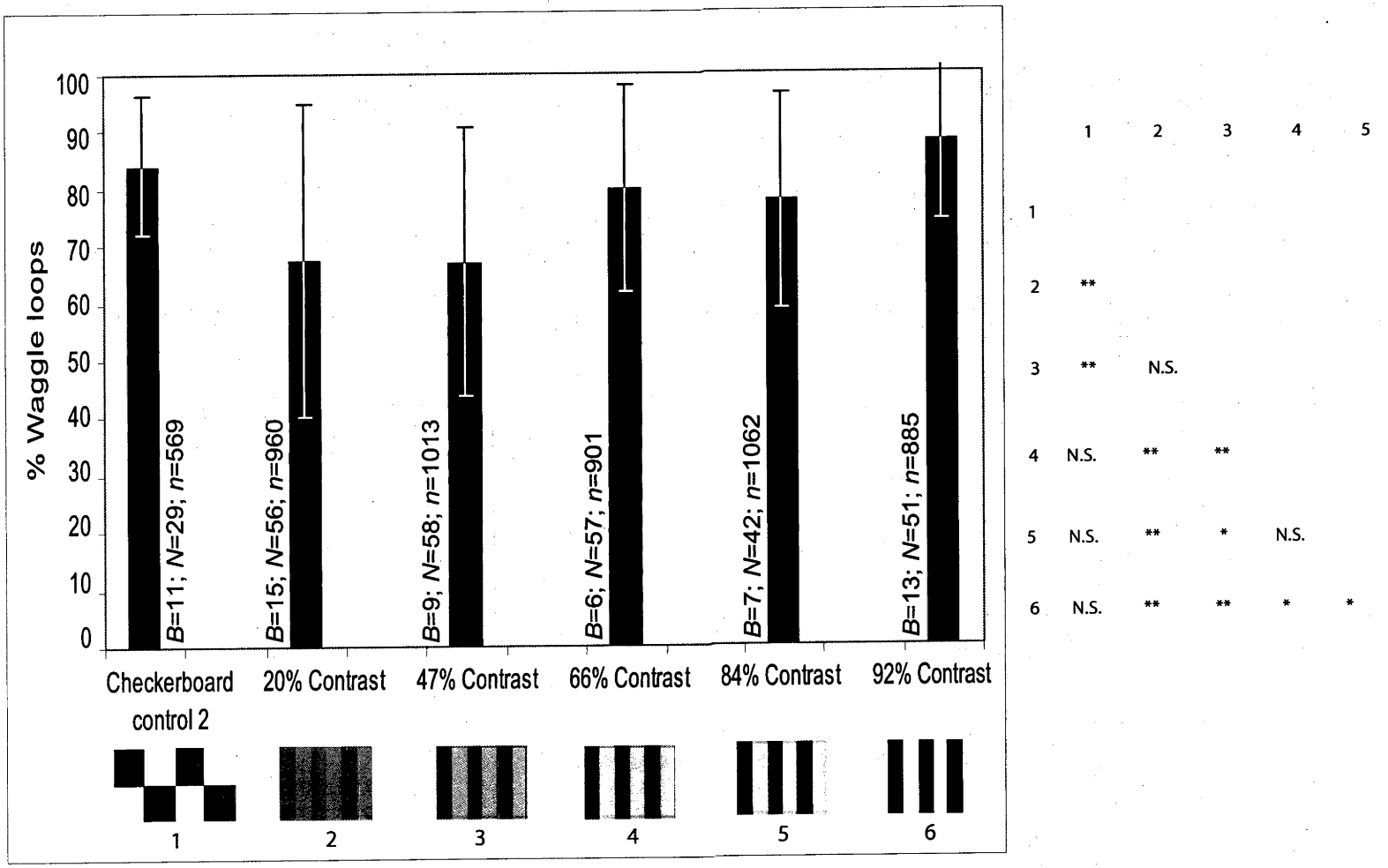


Fig. 4.7. The effect of contrast on the proportion of waggle loops. Other details as in Fig. 4.6.

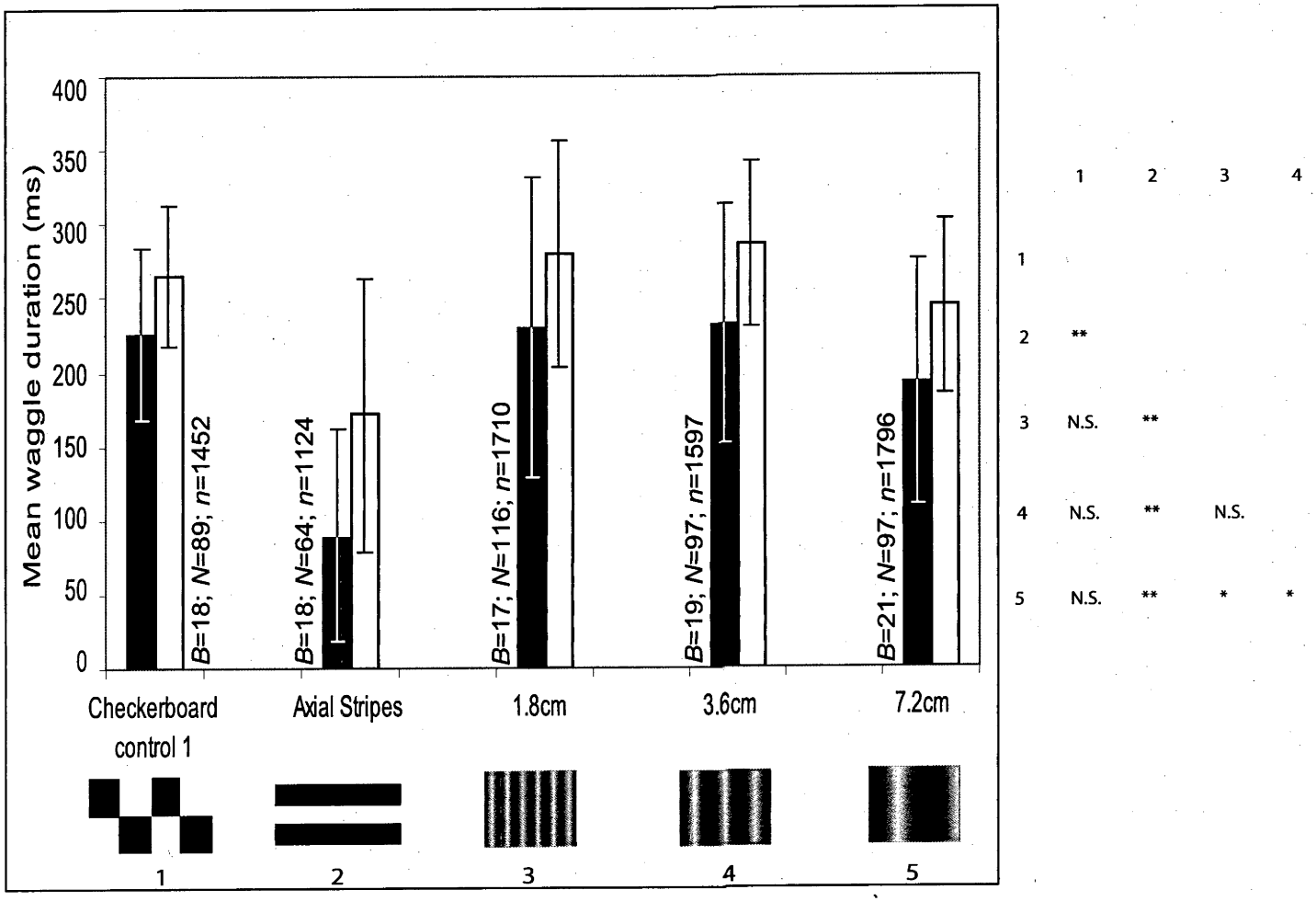
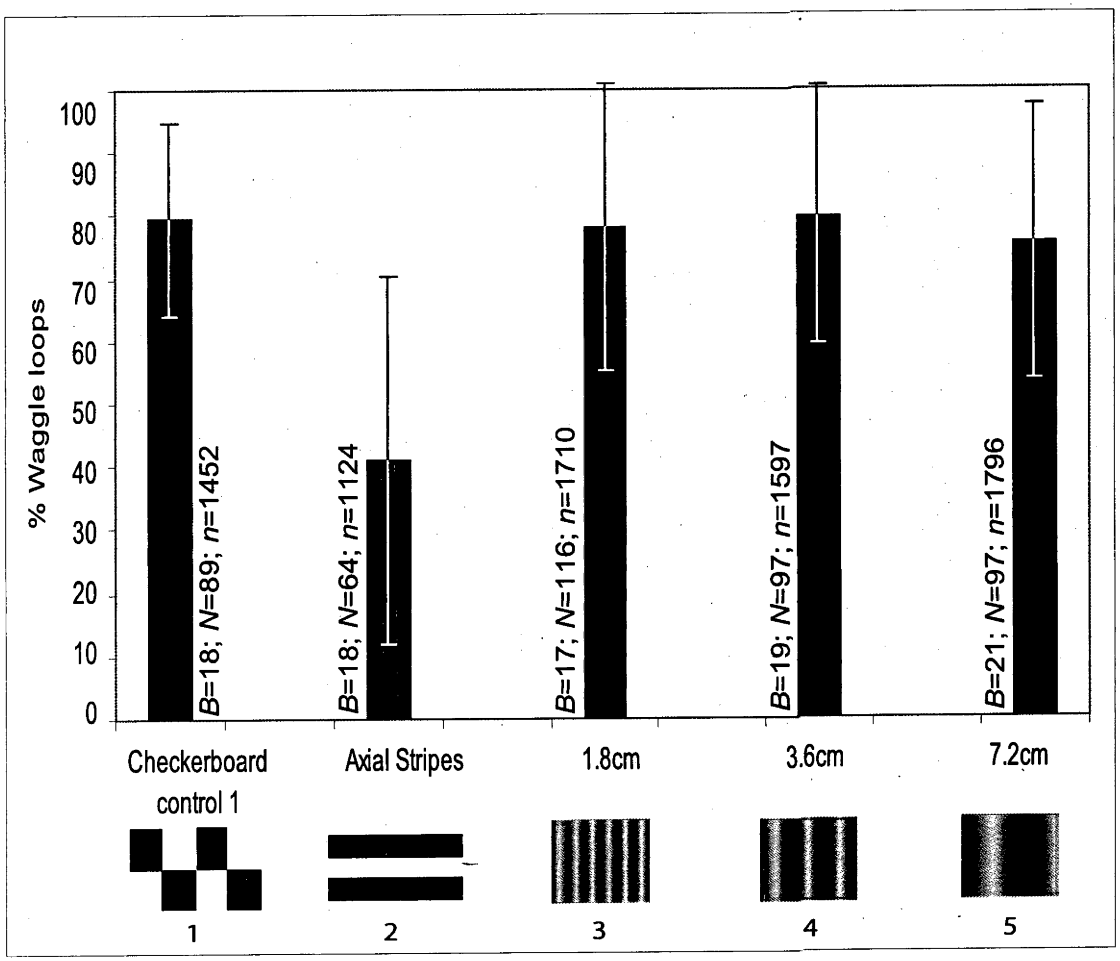


Fig. 4.8. The effect of spatial period on mean waggle duration (black bars) and the mean pure waggle duration (white bars). The walls and floor of the tunnel were lined with sinusoidal gratings of spatial periods 1.8 cm, 3.6 cm and 7.2 cm as shown. A 92% contrast checkerboard pattern (Control 1) and axial stripes (control 2) were used as controls. Other details as in Fig. 4.2.



	1	2	3	4
1				
2	**			
3	N.S.	**		
4	N.S.	**	N.S.	
5	N.S.	**	N.S.	N.S.

Fig. 4.9. The effect of spatial period on the proportion of waggle loops. Other details as in Fig. 4.8.

conditions, however, lies not in their slight differences, but in their *similarity*. Thus, even when optic flow cues were restricted to the floor (and the floor comprised only 20% of the tunnel's interior surface area), the mean waggle duration was still about 70% of the value that was observed when all of the surfaces provided optic flow. The percentage of waggle loops also exhibited a relatively modest variation between these two rather extreme conditions.

When all of the interior surfaces of the tunnel were lined with axial stripes, the mean waggle duration dropped further (to 80 ms, Fig. 4.4), as did the percentage of waggle loops (to 47 %, Fig. 4.5). However, even under this highly impoverished condition, the bees signaled a distance that was greater than that corresponding to the outdoor flight of 35 m to the tunnel entrance (which elicited 33 % waggle loops and a mean waggle duration of 40 ms).

Finally, when the bees returned from an outdoor feeder positioned 41 m from the tunnel entrance, they exhibited 55 % waggle loops and a mean waggle duration of 80 ms. These values represent a flight distance that is slightly larger than that corresponding to an outdoor flight of 35 m, but considerably lower than the distances that were signalled after flights within the tunnel when some or all of the surfaces provided optic flow cues. This is despite the fact that the bees flew the same distance from the hive to the feeder in the tunnel (35 m + 6 m) as they did when they flew to the outdoor feeder at 41 m. Additionally, a flight of 35 m in the open, followed by 6 m in an axial-striped tunnel seems to be equivalent to a 41 m flight in the open, both in terms of mean waggle duration and proportion of waggle loops.

4.4.3 Effect of contrast on the odometric signal

Next, the influence of the contrast of the visual environment on the perception of distance flown was investigated. In a series of experiments, bees were made to fly a constant distance (6m) into a tunnel in which the walls and floors were lined with square-wave gratings of constant period, but with contrasts ranging from 20% to 92%.

Analysis of the dances of bees returning from this tunnel revealed that the odometric signal is rather insensitive to variation of contrast (Fig. 4.6). When the contrast was decreased from 92% to 20% (a 78% reduction), the mean waggle duration decreased from 270 msec to 180 msec (a reduction of only 33%). The 180 msec waggle duration elicited by the 20% contrast grating was more than twice as large as the waggle duration elicited by a tunnel lined with axial stripes (compare with Fig. 4.2). There was no significant decrease in the mean waggle duration when the contrast was reduced from 85% to 66%, or from 47% to 20% ($p>0.05$). The percentage of waggle loops in the dances exhibited a similar insensitivity to variation of contrast (Fig. 4.7). These findings indicate that the odometric signal is rather insensitive to variations of contrast in the environment.

4.4.4 Effect of spatial frequency content of the environment on the odometric signal

In another series of experiments, bees were made to fly a constant distance (6m) into a tunnel in which the walls and floor were lined with sinusoidal gratings of fixed contrast (mean contrast, 58%) but with varying spatial periods of 1.8cm, 3.6cm and 7.2cm. (For a bee flying along the axis of the tunnel, the spatial

frequencies of these gratings as seen by the lateral field of the eye would range from 0.03 cycles/deg to 0.10 cycles/deg.) The results (Fig. 4.8) revealed that the mean waggle duration does not vary significantly with a twofold increase of spatial period from 1.8cm to 3.6cm. There was only a slight, but significant decrease in mean waggle duration with an increase of spatial period from 3.6cm to 7.2 cm. There was, however, no significant difference between the mean waggle durations for any of the spatial periods and those obtained in the conditions in which the walls and floor of the tunnel were lined with a checkerboard pattern (Fig. 4.8; $p>0.05$). But all of these mean waggle durations were significantly higher than that corresponding to the axial-stripe condition, in which there was no optic flow (Fig. 4.8). There were also no statistically significant differences between the waggle loop percentages for the three different spatial periods ($p>0.05$) (Fig. 4.9). In the case of the 92% contrast grating, however (Fig. 4.6), the mean waggle duration is slightly and significantly greater than in the case of the checkerboard or the random texture (Fig. 4.4) ($p<0.05$).

4.5 Discussion

The results of this study indicate that the honeybee's odometer is remarkably robust in its performance. It can be driven by image motion cues that are weak, or restricted to small regions of the visual field. Thus, the odometric signal continues to be strong even when the optic flow is restricted to the walls or the floor of the tunnel. These findings indicate that the honeybee's odometer is remarkably robust to deprivation of optic flow information in large sections of the

visual field, regardless of whether this deprivation occurs in the lateral or the ventral field of the eyes.

At a distance of 8 m into the tunnel, the mean waggle duration is ca. 250 ms. This waggle duration is comparable to that exhibited by bees that return from distances as large as 100m – 200 m in a natural outdoor environment (Srinivasan *et al.*, 2000; Esch *et al.*, 2001). Evidently, the proximity of the walls and the floor of the tunnel greatly amplify the magnitude of the optic flow in comparison with what the bees would normally experience during outdoor flight in a natural environment. These data support the conclusions of earlier studies (Srinivasan *et al.*, 2000, Esch *et al.*, 2001), which presented evidence that honeybees gauge distance flown in terms of the amount of image motion that is experienced by the eyes *en route* to the food source. The present results extend those findings by showing that the odometric signal increases with distance flown in the tunnel, just as it does in the case of outdoor flight in a natural environment. The difference is that in the tunnel the odometric signal increases at a higher rate than during outdoor flight.

The tunnel can thus be used as a convenient experimental device in which to "simulate" outdoor flights of a few hundred metres, and to study the effects of varying the contrast, texture, and other properties of the visual environment on the odometric signal. However, it is possible that even small imperfections in the construction and laying of the axial stripe pattern in the tunnel were responsible in producing small, residual optic flow cues that are registered by the odometer, which are manifest in the waggle loops contained inside dances that are otherwise mostly round.

The data suggest that the walls make a slightly greater contribution to the odometric signal than does the floor. This is consistent with the findings of Srinivasan *et al.* (1997) who reported that bees could locate the position of a feeder in a tunnel more accurately if optic flow cues were provided by the walls instead of the floor. Thus, the combined lateral field of view is evidently more important than the ventral field for odometry. Nevertheless, the honeybee appears to possess a 'flexible' visual system -- one that prefers to use optic flow from the lateral fields of view, but in its absence will use information from other regions. Such flexibility would be advantageous, given that bees forage in a variety of environments that may have extremely different visual properties. It thus appears that, in estimating distance flown, foragers are able to attend only to the strongest optic flow, regardless of where in the visual field the flow originates.

These findings also indicate that the odometric signal is rather insensitive to variations of scene contrast, and that even small contrasts generate sufficient optic flow information to produce an odometric signal of nearly normal strength. The dances elicited by visual contrasts in the range of 66% to 92% are remarkably similar in their properties (Figs. 4.6, 4.7). When the contrast is reduced to below 50%, there is a slight decrease in the mean waggle duration and waggle loop percentage, but these values continue to be high even at a contrast as low as 20 %. This robustness to contrast variations should be of considerable advantage, since the contrast of natural scenes can vary widely and it would be important to have access to a strong and reliable odometric signal even when the contrast in the environment is low. The present result, that there is a measurable odometric signal

even in the axial-striped tunnel (Fig. 4.2, 4.4), suggests that very weak motion cues (such as those from a lake surface) are sufficient to drive the odometer.

The present study indicates that the odometer is also robust to variation in the spatial texture of the visual environment through which the bee flies – provided the texture is capable of providing optic flow information. When the tunnel is lined with vertical gratings, a fourfold change of spatial period produces only a modest variation either in the mean waggle duration, and none at all in the waggle loop percentage (Figs. 4.8, 4.9). A similar insensitivity to variations of spatial period was observed in an earlier study, which investigated the ability of bees to use odometric information to locate a feeder placed at a fixed position inside a tunnel (Srinivasan *et al.*, 1997). There, the bees' accuracy in pinpointing the feeder location was unaffected when the spatial period of the gratings lining the tunnel walls and floor was varied over a four-fold range (Srinivasan *et al.*, 1997). This robustness to variation of spatial texture is also mirrored in the centring response: bees will fly down the middle of a corridor even when the spatial periods of the gratings on the two walls differ by a factor of four (Srinivasan *et al.*, 1991).

It is interesting to note that the dances elicited by the tunnel are fundamentally different from those performed by bees flying in a natural environment to a distant feeder/food source. The proportion of non-waggle loops, when compared to the tunnel dances, is often much reduced or completely absent in dances performed by bees flying in the open (see Chapter 5). This indicates that the dance performed by the tunnel bees is actually a modified form of the waggle dance, possibly elicited as the result of a conflict between the bees' normal

odometric signal derived from optic flow, and the 'true' distance based on the bees' previously acquired knowledge of the environment external to the tunnel.

The effect that the age and/or foraging experience of a worker honeybee might have on her ability to accurately signal a food location is as yet poorly understood. While it has been established, that the deviation in the signalled angle decreases with the age (and therefore experience) of foraging bees (Schweiger, 1958), it is still not clear how the distance component of the waggle dance changes with age. Older studies have reported a decrease in 'dance tempo' as foragers become more experienced at flying to a fixed food source (Schweiger, 1958; see also Von Frisch, 1993, pp. 70-74). The data analysis methods used in these studies, however, would have confounded the true waggle duration (and hence the distance being signalled) with the 'liveliness', and hence the profitability of the food source being exploited (Seeley, *et al.*, 2000). The present study made use of a random selection of foragers leaving the hive entrance, which would therefore have included bees of varying ages and experiences. There is evidence that honeybees possess a General Landscape Memory, that allows localization of multiple places relative to their intended goal, the hive (Menzel *et al.*, 2000). The more experienced bees in the present study are likely to have had a very good knowledge of the environment in the vicinity of the hive. Thus, when they were made to fly into the tunnel with a clear ceiling through which the outside environment is partly visible, there is likely to have been a strong conflict between their position as guaged by external landmarks, such as trees, as opposed to optic flow. It is equally plausible, that very young foragers, or bees foraging for the first time would be more easily 'fooled' by the experimental setup, due to their

limited knowledge of their surroundings. More work clearly needs to be carried out on the development of such a complex behaviour, and the role that age and experience might play on its ontogeny. It would also be interesting to investigate the dance behaviour of bees of known age following flights through the tunnel, and to determine if the proportion of waggle and non-waggle loops changed with age – such an experiment could unfortunately not be carried out due to time constraints. Such considerations were taken into account while deciding on the experimental paradigm for the study reported in Chapter 5. In that study, bees of known age were made to fly in the open to a feeder placed 190 m from the hive entrance, a distance found to induce 'pure' waggle dances in the foragers.

5. Chapter 5: The effects of caffeine on motivation, learning and the acquisition of a complex behavior in the honeybee

5.1 Abstract

Caffeine has long been known to have a wide range of interesting and often familiar behavioural and psychological effects – the vast amount of literature published on this drug every year has significantly improved our understanding of its mode of action, at the behavioural, physiological and biochemical levels. The honeybee has been used as a model organism to assess whether caffeine can influence cognitive performance in an invertebrate organism. Both the motivation and cognitive performance of honey bees trained in a Delayed-Match-to-Sample paradigm are significantly improved in caffeine-treated individuals. Bees were also treated with caffeine shortly after emergence, and observed for several days following the commencement of foraging behaviour. Caffeine was found to chronically reduce the probability of foraging, while simultaneously increasing visit frequency in bees that did forage. The probability of dancing was also reduced in caffeine-treated bees during the final one-third of the observation period. These results suggest that the behavioural effects of

caffeine in the honeybee are similar to, and as complex as those reported in humans and other animals.

5.2 Introduction

Caffeine is arguably the most common psycho-stimulant drug used worldwide and its impact on alertness, memory, mood and general performance in humans is widely acknowledged (Fredholm *et al.*, 1999, Smith 2002). More recently, prior coffee drinking has emerged as the most consistent association with a reduced risk of Parkinson's disease (Schwarzschild *et al.*, 2002). However, the scientific examination of relationship between caffeine and behaviour in humans and other mammals has often produced inconsistent results (Smith 2002, Nawrot *et al.*, 2003). For example, a large number of studies prior to 1990 on the effects of caffeine on more complex cognitive processes failed to detect significant effects in human subjects (Smith 2002). On the other hand, unequivocal beneficial effects on vigilance and cognitive performance in both rested and sleep-deprived individuals have been documented by numerous reports including a study employing a specially developed visual vigilance task (Lieberman 2003). Such contradictions are not unexpected when behavioral effects of a pharmacologically active substance are investigated in a complex, highly interconnected nervous system, but also because of the underlying circuitousness of the path from molecules to behavior. There is therefore considerable interest in developing a simple and efficient animal model system with which to explore the effects of caffeine and other psycho-active drugs on behaviour.

The first part of the present study examines the effects of caffeine on honeybees in a situation where they face a complex cognitive task, the so-called 'Delayed-Match-to-Sample' (DMTS). This paradigm has been used to investigate principles of learning and memory in a number of vertebrate species including dolphins (Herman and Gordon 1972) and monkeys (Salzmann *et al.*, 1993).

The most famous and the best-studied of all honeybee behaviours is arguably the waggle dance. The only known symbolic communication system outside the vertebrate taxon, the honeybee waggle dance requires impressive sensory and motor feats, both on the part of the bees performing the dances, as well as those following the dances (Dyer, 2002). Traditionally, experiments on the waggle dance of the honeybee, be it using bees flying in the open or through narrow tunnels, have used a random subset of foragers exiting the hive at any given time. This group of foragers, trained to an artificial feeder, would include forager bees of all ages and levels of foraging experience. Such an approach would invariably lead to the averaging of a range of very different responses, as it has been shown that certain aspects of the waggle dance change with foraging experience (Schweiger, 1958). The second part of the study explores the effects of caffeine on the initiation of foraging and dancing behaviour and on components of the waggle dance itself, in bees of known age that are observed daily from the time that they commence foraging. This approach will allow the discrimination of age-and experience-related effects on foraging and dance behaviour, from those induced by the pharmacological treatment.

5.3 Methods

5.3.1 Experimental location

5.3.1.1 Maze experiments

All maze experiments were carried out within the All-Weather Bee Flight Facility at the Research School of Biological Sciences, The Australian National University. The only exception was the repeat DMTS experiment, which took place outdoors.

5.3.1.2 Dance experiment

The dance experiment was carried out on an open, gently sloping hillside on the grounds of the Australian National University (Fig. 5.2 a). An observation hive was placed in a small, metal shed on the top of the hill, at a distance of approximately 190 m from the shore of Lake Burley-Griffin (Fig. 5.2 b). The study was carried out during the first two weeks of January, 2004 (summer).

5.3.2 Organism

5.3.2.1 Maze experiments

Bees from a two-box (8 frames each) hive were trained to an artificial feeder, containing 1.5 M sugar solution. The feeder was then gradually moved into the experimental apparatus (Fig. 5.1) in steps of about 20 cm, and in the absence of any visual patterns, in order to teach bees the path to the final, reward chambers. Once the bees had learnt the path to the feeder, the visual pattern to be associated with the reward and the competing pattern were put in place. Caffeine-

treated and control bees were trained to perform a DMTS task by first being made to fly through a 1m-long tunnel, at the entrance of which was placed a sample stimulus (Fig. 5.1). Following the 1-2 second time delay caused by the flight through the tunnel, bees would enter a decision chamber, whose distal end bore two test stimuli, one of which was identical to the sample stimulus. The choice of the matching test stimulus by the bee would lead it to a reward chamber with a feeder containing sugar solution. A similar training protocol was used to train bees in the visual association task, using a Y-maze (see Fig. 3.1). Here, bees would have to learn a single visual stimulus, which was always associated with a reward of sucrose solution.

A 2 μl drop of 100 mM caffeine dissolved in di-methyl formamide (dMF) was placed on the thorax of each bee to be treated (while drinking from the feeder) prior to training. Control bees were given only a 2 μl drop of dMF. Bees were treated after they had learnt to fly through the maze and find the feeder, but before being trained with any stimuli. The dose administered is not directly comparable to quantities of caffeine used in experiments with vertebrate animals or to human consumption because the efficiency of cuticular penetration is not expected to be 100%. However, it was reasoned that in insect behavioral studies a non-invasive topical delivery is far superior to injections that often lead to increased mortality and/or microbial infections (Kucharski and Maleszka 2003). Indeed, the survival of caffeine treated bees was not different from that of the untreated ones. The choice of dosage for caffeine was based on the results of a dosage-dependence experiment involving the proboscis extension reflex (PER, see Chapter 2 for details on methodology) paradigm. A concentration of 100mM

brought about the highest improvement in cognitive performance of very young (4-day old) bees, and was also very well tolerated, with low levels of mortality (5-10%) (Maleszka and Helliwell, unpublished data).

5.3.2.2 Dance experiment

A frame of wax comb containing brood cells was removed from an experimental beehive, and placed in an incubator at 31°C and 80% humidity overnight. The following morning, approximately 300 of the newly-emerged juvenile worker bees were collected and held in mesh cages. Bees were narcotised on ice in groups of 10, and while immobilised, were individually marked with plastic numbered (and coloured) tags (Opalithplättchen, Bienen-Voigt und Warnholz) (Fig. 5.3). 2 μL of 100 mM caffeine was also administered to half the narcotised bees, immediately following the marking procedure – the remaining half received 2 μL of the solvent dimethyl formamide (dMF). The bees thus treated were allowed to recover, before being introduced into a 4-frame observation hive at the field site.

5.3.3 Data collection and analysis

5.3.3.1 DMTS experiment

Two separate experiments were carried out with two different hives; each time, a new set of bees was treated and trained. Equal numbers of caffeine-treated and control bees (~15) were marked and treated at the beginning of each experiment. All bees used in the DMTS and paradigm were given individual paint markings to aid in their identification during the process of data collection. The

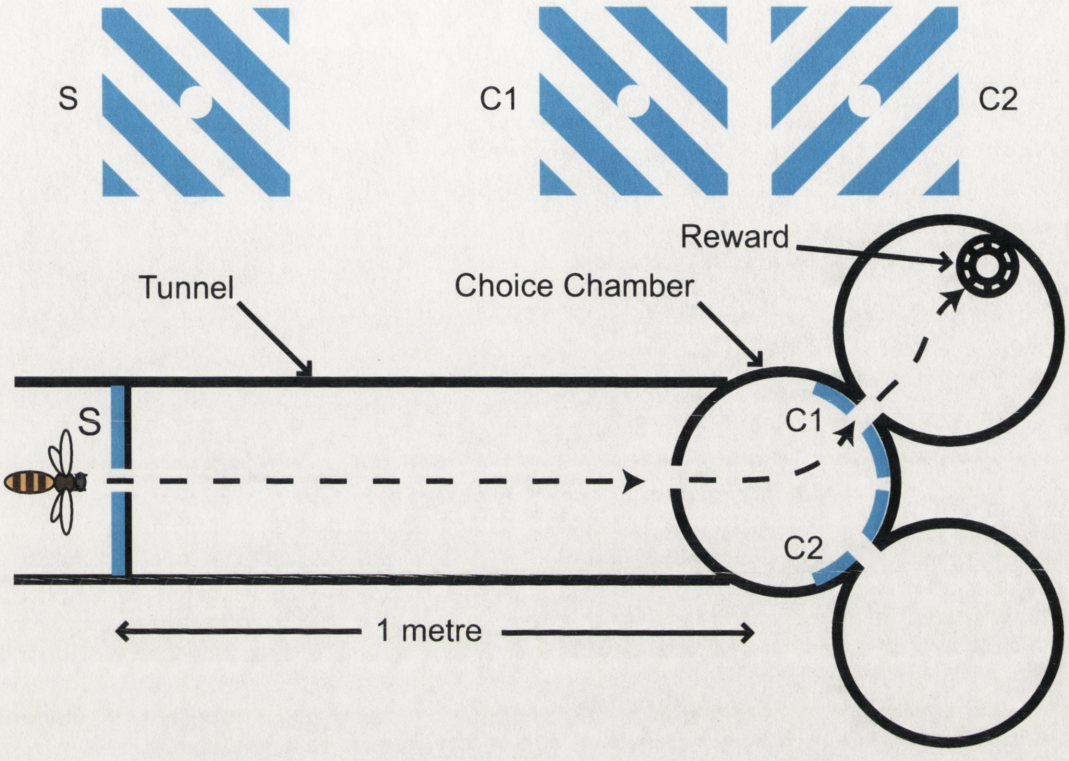


Fig. 5.1. Layout of the Delayed Match-to-Sample (DMTS) experimental apparatus. The bee encounters and flies through the initial sample pattern (S) before traversing a 1m-long tunnel. Upon entering the choice chamber, she is presented with two choice patterns (C1 and C2), only one of which (C1 in this case) is identical to S. The bee must choose the matching pattern C1 in order to obtain a reward of sugar solution.

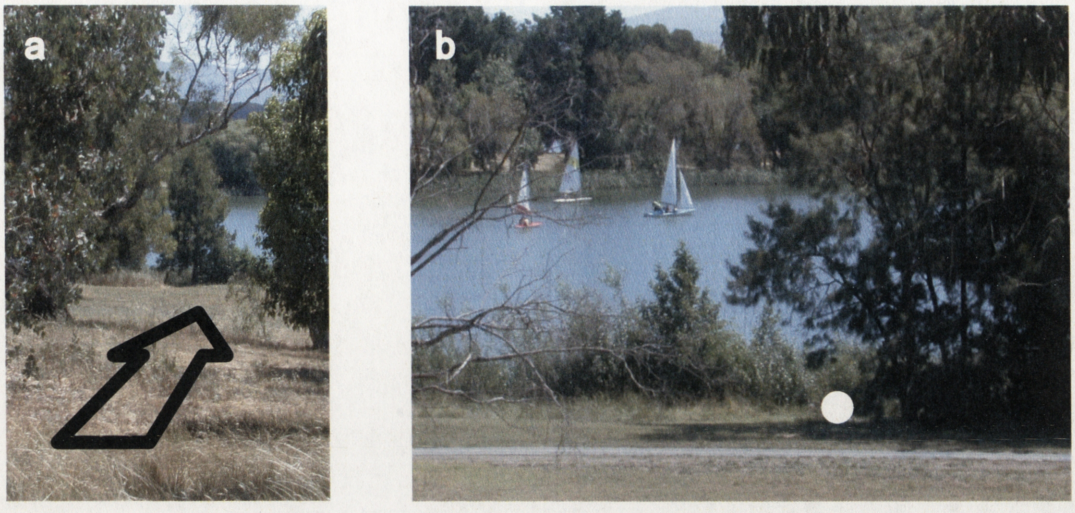


Fig. 5.2. The field site where the dance experiment was carried out. a) A view of the flight path down to the lake shore; the picture was taken immediately outside the hive entrance. b) The shore of Lake Burley-Griffin. The white dot marks the location of the experimental feeder.

Fig. 5.3. Numbered caffeine-treated and control bees at the experimental feeder on the final day of data recording. Practically all the foragers at this stage are experimental bees. Three recruits of unknown age (marked with blue paint) can also be seen.

first choices of bees entering the decision chamber were recorded. The proportions of correct choices were pooled for all bees in each category (*i.e.* caffeine-treated and control) to obtain a final percentage. The visit frequencies of caffeine-treated and control bees were also monitored and recorded. Twelve 20-minute training sessions were completed, each comprising two 10-minute blocks, where the feeder position was alternated. χ^2 Tests were carried out to test for statistical significance.

5.3.3.2 Y-maze experiment

Two separate experiments were carried out with bees from a single hive. However, different sets of bees were used for each experiment. All bees were given individual paint marking to aid identification and data collection. The first choices of bees entering the decision chamber were recorded. The proportions of correct choices were pooled for all bees in each category (*i.e.* caffeine-treated and control) to obtain a final percentage. Seven 20-minute training sessions were completed, each comprising two 10-minute blocks, where the feeder position was alternated. Learning curves for the two conditions were generated, based on the scores from the training sessions. Long-term memory for both groups was tested in a 10-minute retention test without a reward at 4 days and 8 days after training.

5.3.3.3 Dance experiment

The bees that had been introduced into the observation hive were periodically checked for signs of foraging behaviour. In this particular case, no

significant foraging activity was observed from the experimental bees until approximately 12 days after their emergence. On the 12th day, a feeder with sucrose solution was placed outside the hive entrance, in order to attract bees to it. Once about 20 bees from the observation hive had found the feeder, it was gradually moved further and further away, downhill and in the direction of the lake's shore. The feeder reached its final position approximately 190 m from the hive entrance at noon the following day (Day 13).

The recording of dances at the hive and visits to the feeder were started on the afternoon of day 13, and continued every day till day 23. Data collection could not be carried out on days 14 and 15 due to unfavourable weather conditions. On days when data were recorded, the feeder would be placed at the final 190 m position, and any dances taking place at the hive would be recorded over a 1-2 hour period. Another investigator would remain during this entire period at the feeder position, and simultaneously record all visits by the marked, experimental bees. Bee numbers were controlled at the feeder by killing any excess, unmarked foragers and recruits. Bees were also prevented from locating any other (natural) food sources by killing, at the hive entrance, any unmarked returning foragers. Marked bees were observed at the hives for about 15 minutes each day before the feeder was placed out, to determine if any other food sources had been located, and were being signalled. Dances were later analysed in the lab in the manner described in Chapter 4, using the dance analysis program 'Sambee', developed by Sylvain Forêt (RSBS, ANU)

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5.4 Results

5.4.1 DMTS study

In accord with previous studies on learning in honeybees (Zhang *et al.*, 1999, Giurfa *et al.*, 2001), the bees in our study were also able to successfully learn the DMTS task *i.e.*, the percentage of correct responses was significantly greater than a random-choice score of 50% ($p<0.001$) (Fig. 5.4 a). However, it was the caffeine-treated bees that performed significantly better than the control bees (71% and 65% correct responses respectively, $p<0.05$). In addition, we observed that the total number of visits to our experimental apparatus over a two-day period was much higher for the caffeine-treated bees than in the case of the controls (585 and 391 visits respectively, $p<0.001$) (Fig. 5.2 b). This was in spite of the fact that equal numbers of bees (~15) were marked and treated for each group before the start of the training procedure.

The enhanced performance of the caffeine-treated bees could have been due to their greater number of visits to the experimental apparatus (and hence more practice in performing the task). To determine if it really was the drug treatment that was improving the bees' performance, we compared the performance of the two groups after an equal number of visits (*i.e.*, 391 visits each) (Fig. 3 c). Under these conditions, the caffeine-treated bees were found to be performing even better (75% correct, $p<0.01$).

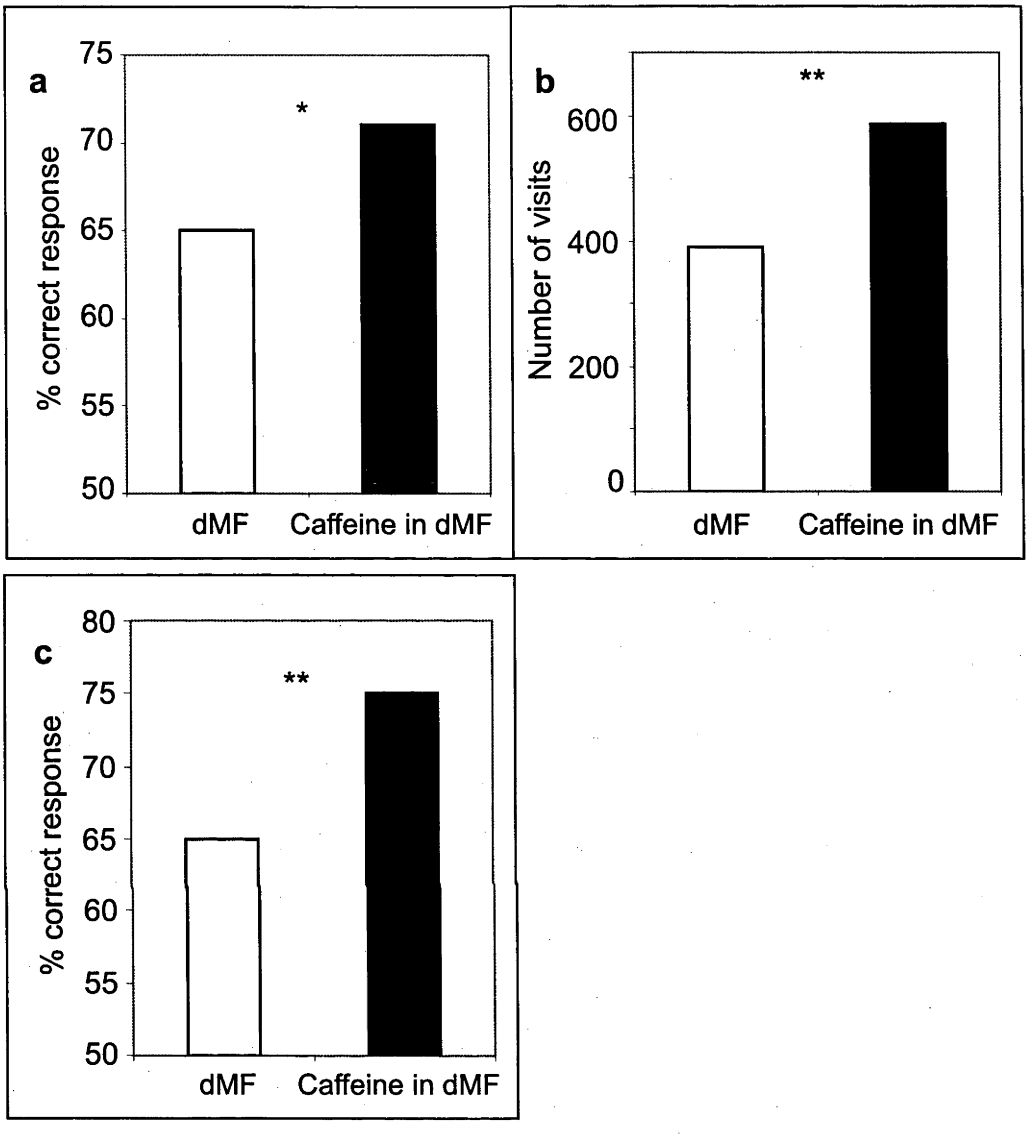

Fig. 5.4 a) Caffeine-treated bees perform significantly better than controls at the DMTS task (* $p<0.05$, χ^2 Test). The figure shows the results obtained at the end of the experiment, following differing numbers of visits from the treated and control bees (see Fig. 5.4 b). b) Caffeine-treated bees visited the apparatus much more frequently than controls during the course of the experiment (** $p<0.001$, χ^2 Test). c) Caffeine-treated bees are found to perform even better when the number of visits for both groups is equalised. The experiment was carried out twice, once indoors in the climate-controlled All Weather Bee Flight Facility at the RSBS, and then repeated outdoors, using a different hive. The results obtained from both experiments showed a similar trend. Fig 5.4 shows data from the first (indoor) experiment.

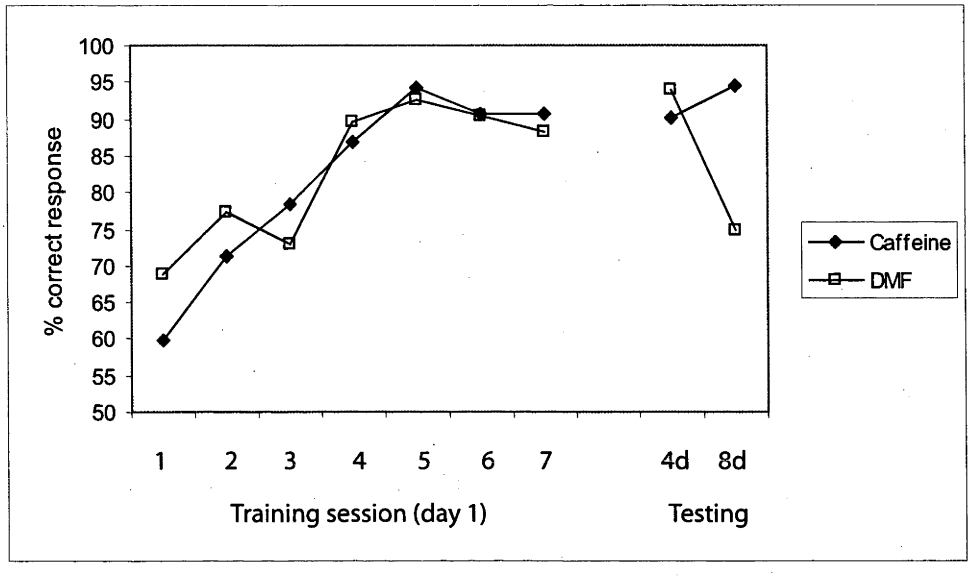

Fig. 5.5. Learning curve obtained from training forager bees in a visual association in a y-maze. Bees were subjected to seven 20-minute sessions on Day 1, followed by 6 days of 'forgetting' time. The same bees were then tested for the long-term retention of visual memory on days 7 and 8. No statistically significant differences were recorded (χ^2 Test).

5.4.2 Y-maze study

The learning curves for the y-maze visual association task were not significantly different for caffeine-treated and control bees. Both sets of bees attained a maximum performance level of ~90% after just five 20-minute training sessions. The performance of bees on day 7 was unchanged, with both groups scoring >90%. On day 8, the control bees displayed a drop in performance, while the caffeine-treated bees' score was as high as ever. The fall in performance was not significant, however, due to the low numbers of experimental bees still visiting the feeder on the last day.

5.4.3 Dance experiment

Caffeine treatment of worker honeybees at eclosion brought about marked and sustained changes in foraging behaviour later in adult life. Caffeine treatment consistently reduced the total number of bees foraging on all data-collection days (Fig. 5.6), possibly as a result of toxicity brought about by a too-high concentration of drug. Although the probability of foraging increased with increasing age, there were always more control bees at the feeder than caffeine-treated ones. Another important effect of caffeine was the consistent increase in visit frequency (Fig. 5.7). Caffeine-treated bees visited the feeder at a higher rate than control bees, except on days when they were re-treated with caffeine (Fig. 5.7).

At the hive, it was found that bees visiting the feeder had roughly equal probabilities of performing dances, regardless of whether they had been treated with caffeine or not ($p>0.05$, χ^2 Test) (Fig. 5.8). Dance probability was measured

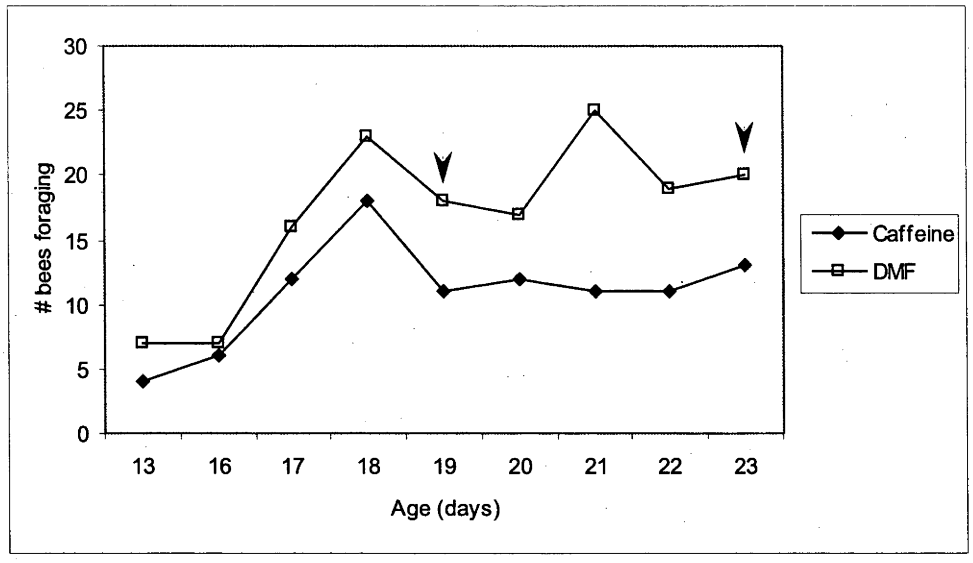

Fig. 5.6. The effect of caffeine on the probability of foraging. The bees were treated with 100 mM caffeine in dMF at birth (or with dMF as a control), and on days 19 and 23 (arrowheads). Date could not be collected on days 14 and 15 due to bad weather.

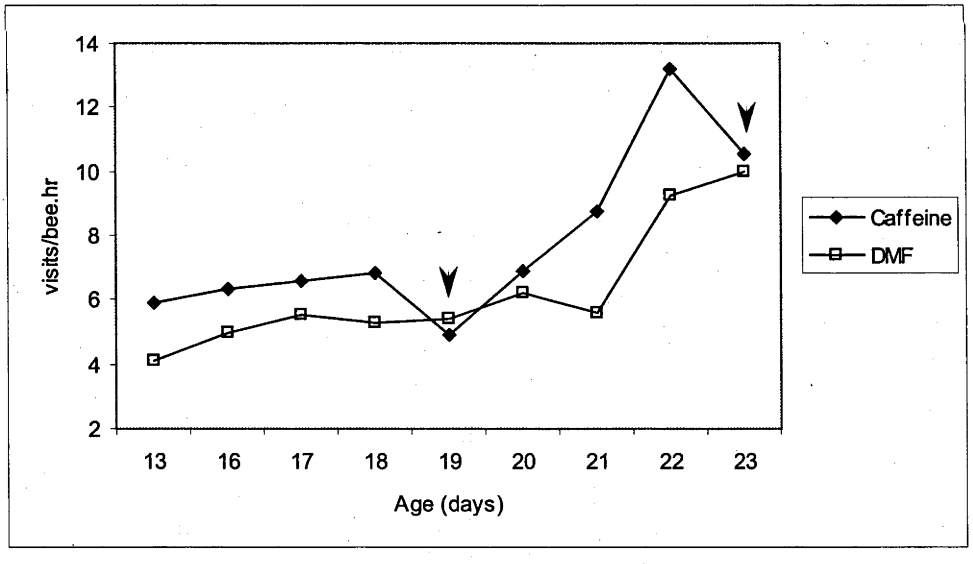

Fig. 5.7. The effect of caffeine on visit frequency. Other details as in Fig. 5.6.

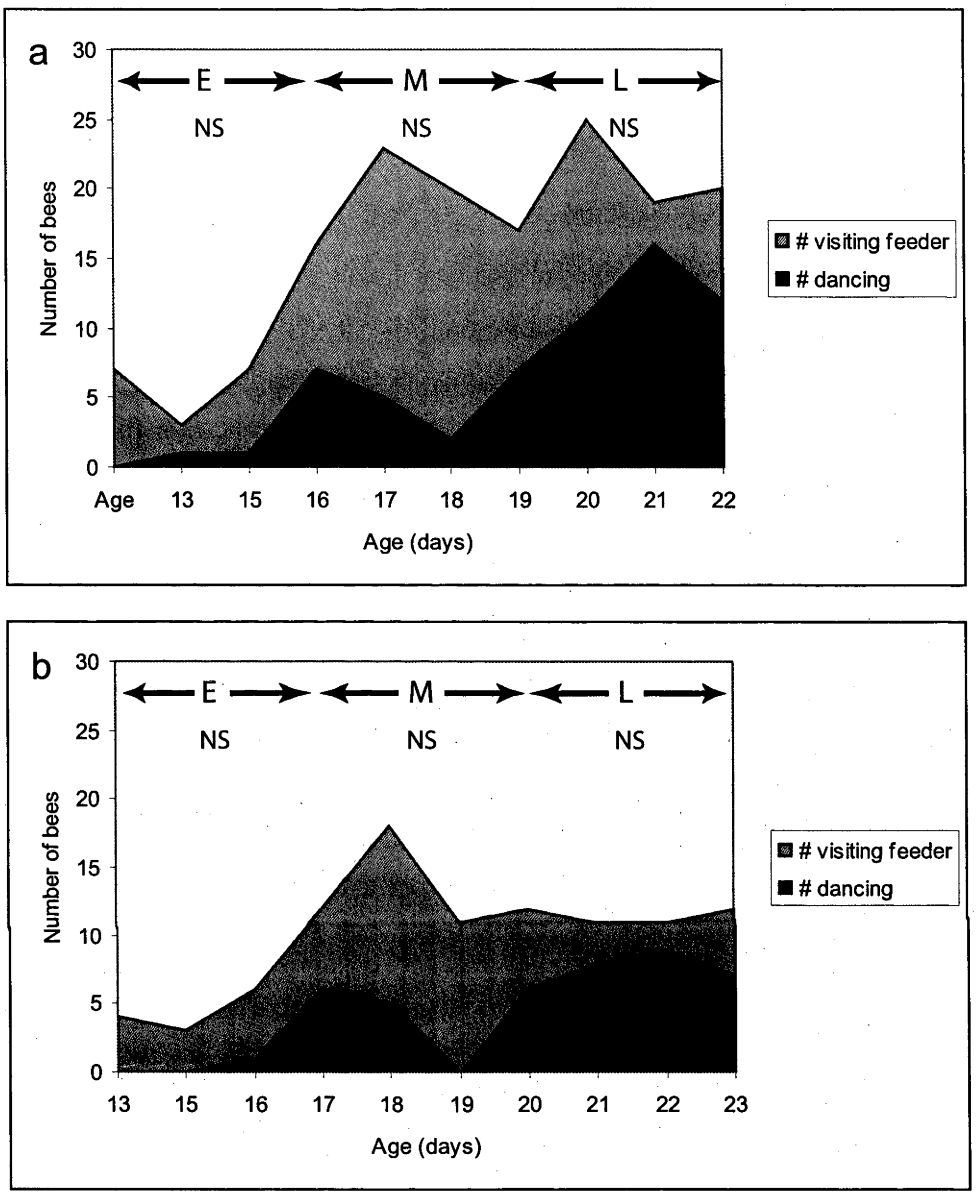

Fig. 5.8. The effect of caffeine on the probability of dancing. The graph shows the daily proportion of new labelled bees appearing at the feeder, that return to the hive to later perform dances. a) DMF control bees; b) caffeine-treated bees. The number of bees visiting the feeder corresponds exactly to the data presented in Fig. 5.6. Data were grouped arbitrarily into 'early' (13-17 days), 'middle' (18-20 days) and 'late' (21-23 days) for the purpose of statistical analysis. NS indicates no significant difference ($p>0.05$, χ^2 Test) between the caffeine-treated and DMF data.

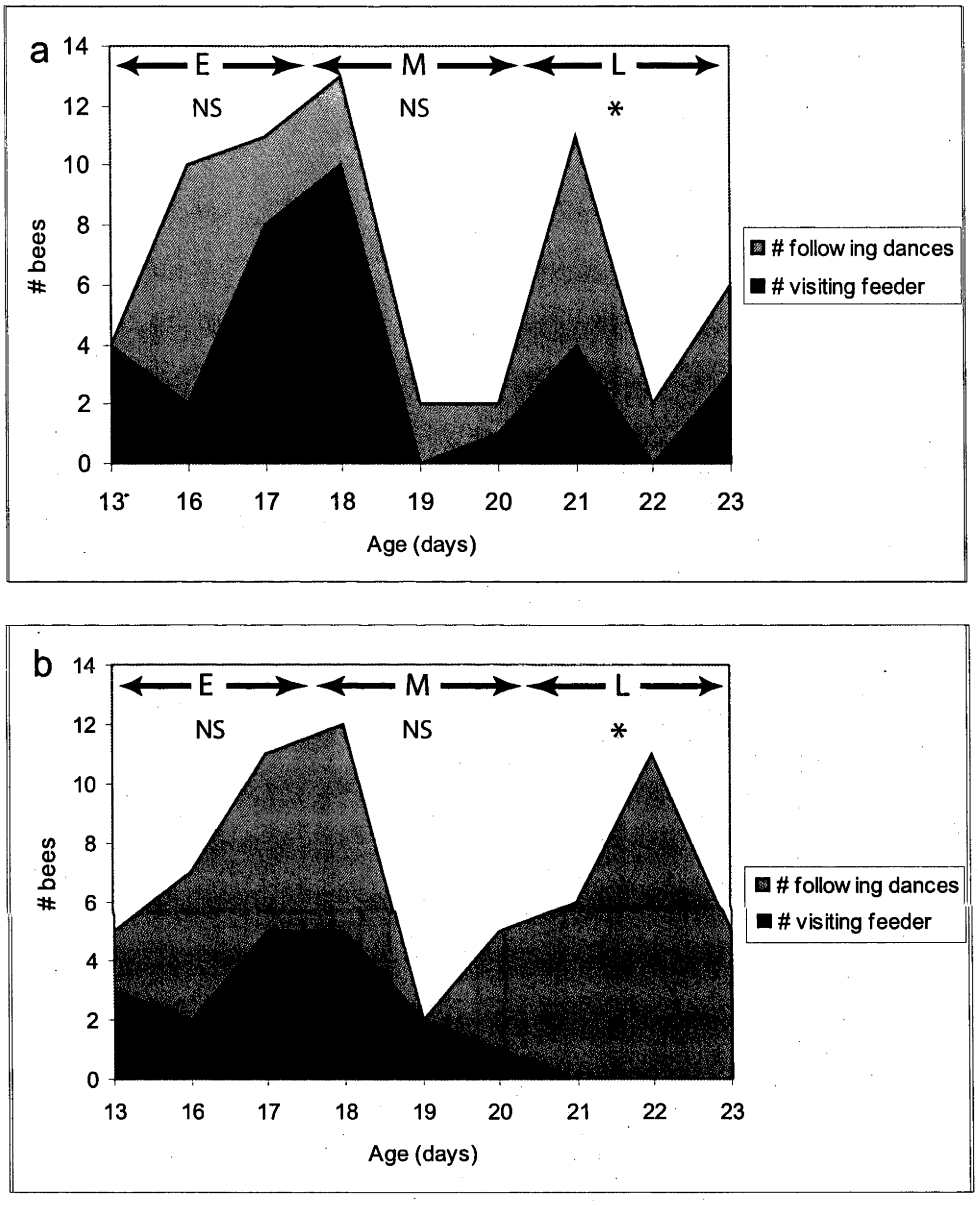

Fig. 5.9. The effect of caffeine on the probability of being recruited to the food location being signalled. The graph shows the daily proportion of new bees observed to be following dances at the hive, that later fly out of the hive to look for (and find) the experimental feeder. a) DMF control bees; b) caffeine-treated bees. The bees visiting the feeder in this case form a subset of the bees represented in Fig. 5.6. Other details as in Fig. 5.8. * indicates a significant difference at $p<0.01$ (χ^2 Test) between the caffeine-treated and control bees.

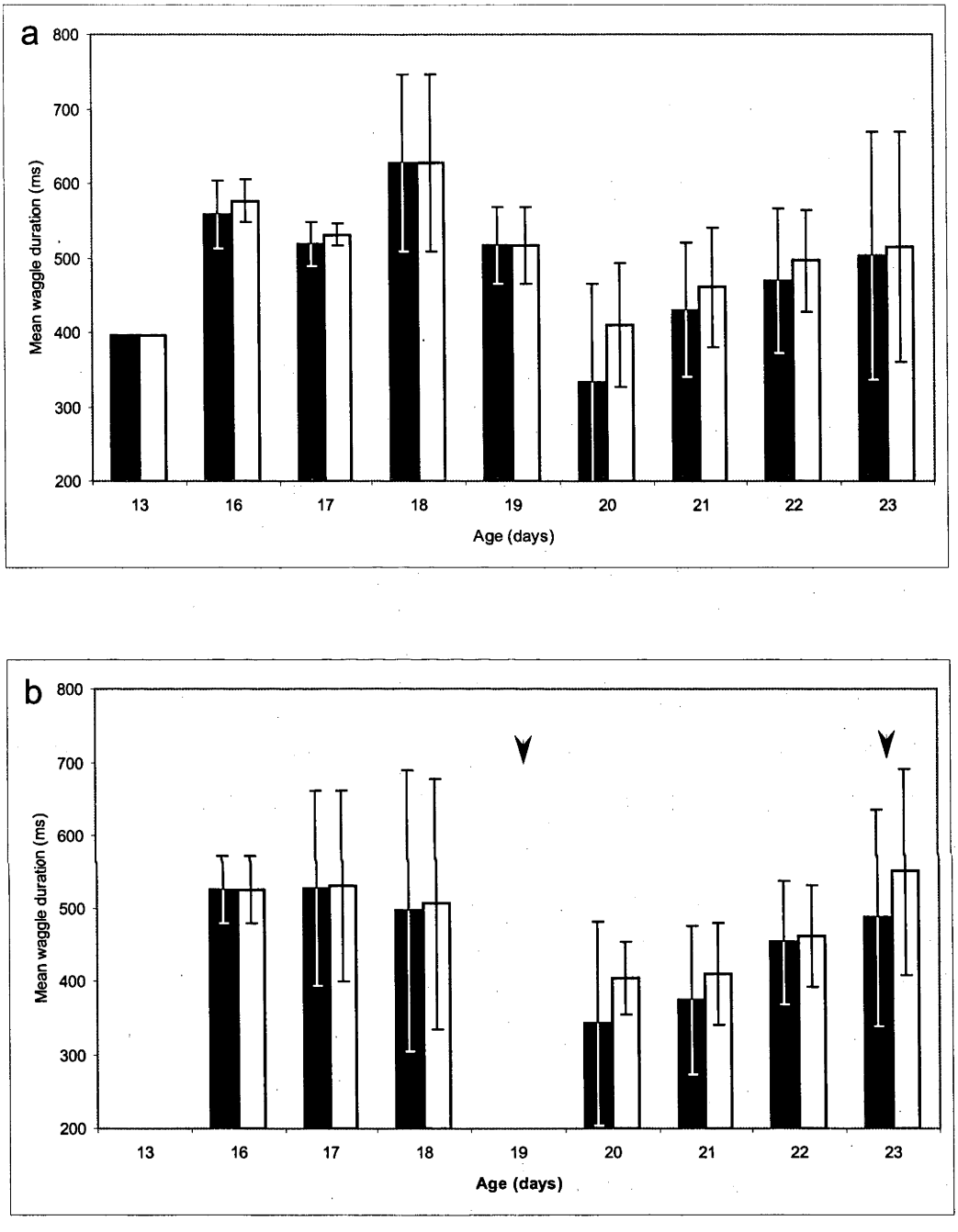

Fig. 5.10. The effext of age on the mean waggle duration (black bars) and the mean pure waggle duration (white bars) of a) DMF control bees and b) caffeine-treated bees. Other details as in Fig. 5.6.

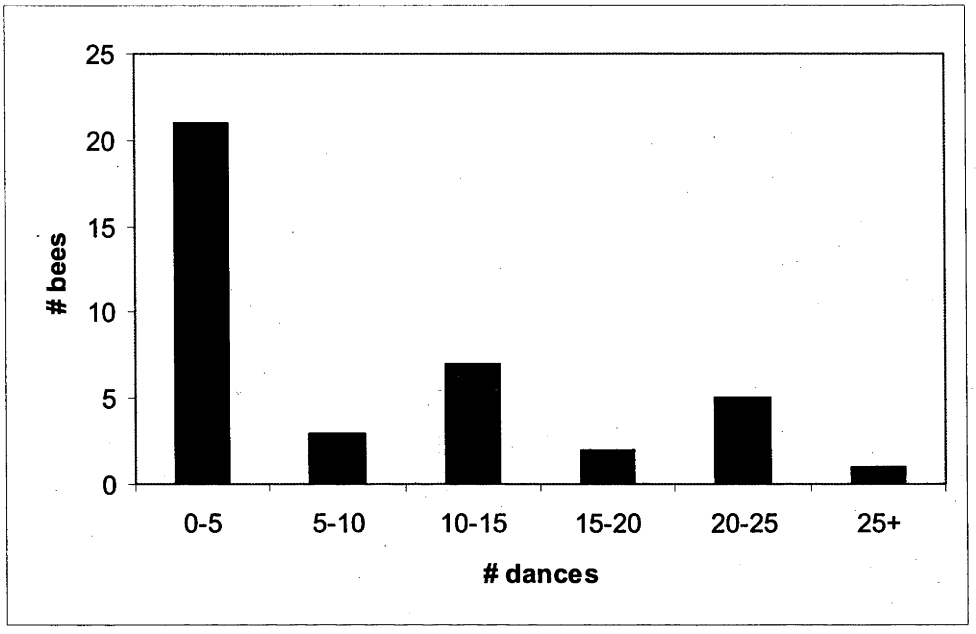

Fig. 5.11. Numbers of bees arranged according to the frequency of dances performed during the observation period. Most bees performed <6 dances over the experimental period, whereas very few performed >20.

as the proportion of marked bees recorded at the feeder, that later return to the feeder to dance the same day. Age was found to strongly influence dancing probability, which showed a significant decrease during the 21-23 days period ($p<0.005$, χ^2 Test, not shown). This was true for both caffeine-treated, as well as control bees. In keeping with the reduced foraging probability shown in Fig. 5.6, it was also found that caffeine-treated bees, that followed the dances of other bees indicating the feeder location, were much less likely to venture out of the hive and search for the food source ($p<0.01$, χ^2 Test) (Fig. 5.9). Age again played a role in regulating foraging frequency, but only for the caffeine-treated bees ($p<0.005$, χ^2 Test, not shown). No significant effect of age could be determined for the control bees ($p>0.05$, χ^2 Test).

An age-dependant trend in the waggle durations for either group of bees could only be observed on the last four days, when there was a steady increase in mean waggle duration (Fig. 5.10). A striking observation was the complete suppression of dancing in caffeine-treated bees on day 19 (a day of re-treatment). The accuracy in the signalling of direction also showed no obvious trend (not shown). In addition, the proportion of non-waggle loops in all dances was found to be very low (mostly <10%) across all ages.

A surprisingly small number of bees (6 in total, or 15% of the experimental bees) was responsible for a large proportion (~50%) of dances performed during the sampling period, while almost half the experimental bees performed very few (~13%) of the total dances recorded (Fig. 5.11). The dose of caffeine used was not found to have any effect on the mortality of treated bees, as

roughly equal numbers of control and treated bees were present in the hive at the end of experimentation each day.

5.5 Discussion

Caffeine might play a direct role in the improvement of the DMTS task by increasing the level of alertness or cognitive arousal in bees, as has been shown to occur in humans (Herz 1999, Brice and Smith 2001, Ryan *et al.*, 2002). At another level, the improved learning seen in the caffeine-treated bees might be a result of increased motivation brought about by the drug. A significantly higher frequency of visits to the experimental apparatus is likely to result in more reinforcements leading to enhancements in the encoding of new information. This result is reminiscent of human studies showing that caffeine improves encoding of new information and counteracts the fatigue that develops over the test session (Smith *et al.*, 1999). Increased reinforcement is unlikely to be the main cause of the improved performance seen in the caffeine-treated bees, however, as equalising the number of visits for both group also led to a (even more) pronounced increase in the performance of the DMTS task.

The effects of caffeine on motor performance in humans are well known (reviewed in Garrett and Griffiths, 1997; Lorist and Tops, 2003), and may account for several of the phenomena reported in the present study. Caffeine induced almost a 50% increase in the visit frequency of bees to the DMTS task, and also chronically elevated the visit frequency of bees throughout the sampling period of the dance experiment. Paradoxically, however, treatment with caffeine also

reduced the probability of foraging during this period, suggesting a complex mode of action for this drug in the honeybee brain. Indeed, the behavioural effects of caffeine in humans are not entirely consistent: negative or null effects on motor performance have been reported, which may be a result of complicated interactions between the stimulant actions of caffeine and the arousal level of subjects and the nature of task requirements (Lorist and Tops, 2003).

The y-maze experiment in the present study showed that acquisition of a visual association task is not affected by caffeine administration. The DMTS paradigm (requiring the learning of the 'matching rule', as well as temporary storage of the initial stimulus in short term memory at each trial) is a much more challenging task, and therefore not directly comparable to the y-maze paradigm. The nature of the DMTS task, however, would more likely allow any increase in alertness and cognitive arousal, brought about by caffeine, to lead to an improvement in performance. Control bees in the y-maze experiment showed a large, but non-significant decline in the long-term retention of visual associative memory. The number of bees remaining at this late stage of the experiment was low - this could account for the non-significance of the result. Further testing needs to be carried out to determine caffeine's effect on long-term memory in honeybees.

The probability of foraging, the frequency of visits to the feeder, as well as the probability of dancing were found to increase with age in both groups of bees (caffeine-treated and control). No obvious effect of caffeine could be observed on either the length of the waggle duration or the precision in the indication of direction on bees of any age. A striking effect, however, was the complete

suppression of dance behaviour on the first day of caffeine re-treatment (when the bees were 19 days old, Fig. 5.8 b). This decline in dancing corresponded with a fall in the frequency of visits to the feeder; a similar reduction in visit frequency was observed following caffeine re-treatment at 23 days (Fig. 5.6). Caffeine re-treatment also brought about an abolition of the recruitment of new experimental bees to the feeder, in contrast to the control bees, which were being recruited even on the last day of data collection. The stimulatory effect of caffeine on the visit frequency of treated bees is therefore surprising in the light of these inhibitory effects on foraging and dancing behaviour. It appears that the concentration of caffeine used in this study has markedly different acute- and chronic-phase effects on behaviour: the acute effect appears to be an inhibitory one, which induces shock-like symptoms in treated bees, and causes them to temporarily remain within the hive with low levels of activity. This soon wears off, however, and is replaced by the chronic, stimulatory effect, which causes bees to visit the feeder more frequently. Higher does of caffeine have been shown to suppress physiological functions such as respiration in vertebrates (Howell and Landrum, 1994), and it is conceivable that similar phenomena should be occurring in the honeybee.

Age was found to not have a marked effect on the signalling of either the distance or the direction of the food source. This may be partly due to the very gradual increase in foraging activity over the observation period. As a result, very few bees were available during the first few days of observation, and some new recruits that had never danced or foraged before were observed at the feeder even on the last two days. The probability of dancing also increased gradually, leading

to a shortage of dance data early in the bees' foraging careers. This experiment will clearly have to be repeated, possibly with larger numbers of bees, for a more accurate assessment of ontogenetic changes in dance behaviour.

Recent data suggest that the stimulating effect of caffeine on behavior is caused by a feedback loop in the nerve cells (Vaugeois 2002). Caffeine blocks the A2A adenosine receptors leading to a cascade of events involving adenylyl cyclase, cAMP, protein phosphorylation and gene transcription. At the neurotransmitter level, a number of systems may be affected including dopaminergic and cholinergic transmissions (Schwarzschild *et al.*, 2002). At the cellular level, adding caffeine to hippocampal slices leads to calcium release from internal stores and the growth of new dendritic branches (Korkotian and Segal, 1999).

While the effects of caffeine on the honeybee nervous system remain to be investigated, it is certainly possible that at the molecular level, caffeine in the honeybee acts in a manner similar to that in mammals. A recent microarray-based study revealed that caffeine induces transcriptional changes in heads of adult bees (Kucharski and Maleszka 2002). In addition, the highly conserved adenosine receptors that are encoded by the honeybee genome lend further support to this idea (Maleszka unpublished).

In conclusion, the cognition- and activity-modulating effects of caffeine in the honeybee suggest that this drug can be used as a powerful tool to investigate general principles for the organization of behaviour in this species. In addition, the remarkable similarity in behavioral effects of caffeine between a simple invertebrate and complex mammals suggests that non-invasive drug treatments

that modify behaviors in an easily manipulable insect system can be explored to advance our understanding of the complexity of human behavior.

6. Chapter 6: Gene expression changes accompanying pharmacological treatments, as determined by real-time RT-PCR

6.1 Abstract

Real-time RT-PCR was carried out on brain tissue from young worker honeybees, that had been subjected to a variety of pharmacological treatments. Previous chapters of this thesis have reported behavioural changes brought about by the administration of drugs targeting the glutamergic system, as well as caffeine. Genes involved in glutamergic synaptic transmission were therefore investigated, and up to ~3-fold changes in the expression of some genes were detected. These differences are discussed in the context of honeybee ontogenetic development, and vertebrate models of glutamergic transmission

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6.2 Introduction

The recent surge of interest in the molecular basis of all things biological has, not surprisingly, made a profound impact on the field of honeybee behaviour as well. Driven by the astonishing progress in molecular biological technology,

the desire to understand the molecular and genetic underpinnings of what is perhaps the world's best-studied social insect has culminated in the realisation of the Honeybee Genome Project (2004). While the molecular correlates of various aspects of behaviour, such as learning and memory, are only just becoming apparent, several exciting and intriguing results have nevertheless been published in the last few years. Hitherto poorly understood phenomena, such as the age-related division of labour and the onset of foraging have been shown to be regulated by changes in the expression levels of the *period* gene, which is well known for its role in circadian rhythms (Toma *et al.*, 2000) and the *foraging* gene, which encodes a cGMP-dependant protein kinase (Ben-Shahar *et al.*, 2002). Similarly, a decrease in acetylcholinesterase gene expression has been found to accompany the switch from 'nurse' to 'forager' behaviour in honeybees (Shapira *et al.*, 2001).

The advance of microarray technology has allowed researchers to assess the complex pattern of gene expression changes that accompany phenomena, such as the response to a treatment or the attainment of a developmental milestone. It is now possible, for instance, to obtain an overview of the genes affected by the administration of pharmaceutical agents, such as caffeine (Kucharski and Maleszka, 2002), or by exposure to ecologically relevant stimuli, such as the Queen Mandibular Pheromone (Grozinger *et al.*, 2003). Such techniques have uncovered a surprising amount of genomic plasticity in the bee brain, which has the potential for being used to predict behavioural trends (Whitfield *et al.*, 2003).

Real-time Reverse-Transcription Polymerase Chain Reaction (RT-PCR) is yet another method that can be used to semi-quantitatively investigate changes in

the expression of individual genes. This technique has been used to reveal that L-glutamate administration to neonatal rats can bring about profound changes in the expression of genes such as growth hormone and insulin-like growth factor (Kovacs *et al.*, 2000), and various NMDA receptor subunits (Beas-Zárate *et al.*, 2001, 2002) (See Section 1.2.1). Real-time RT-PCR allows one to assess the relative amounts of messenger RNA (mRNA), and hence the expression of a particular gene, present in tissue obtained from control and treated animals. The data can also be normalised using the relative expression levels of a reference gene (usually a housekeeping gene), whose expression level is known to be unaffected by the treatment in question (*e.g.* Pfaffl, 2001). This method of analysis allows the standardisation of each reaction run with respect to RNA integrity, sample loading and inter-PCR variations.

Real-time RT-PCR was used in the present study to investigate any possible changes in the expression of a handful of genes over a wide range of treatments. These include the topical application of the insect hormone Juvenile Hormone (JH), the JH analogue methoprene, the feeding of memantine, L-glutamate and aspartate in honey, and the topical application of caffeine. While the role of JH as a gonadotropin in honeybees and other eusocial insects is still being debated, there is strong evidence for its function as a 'behavioural pacemaker' (Robinson and Vargo, 1997). JH levels in the hemolymph of worker bees correlate closely with the behavioural status (*i.e.* nurse or forager) of the animal. In addition, even precocious foragers and old nurses have the same JH titres in their hemolymph as their chronologically 'normal' counterparts (Robinson *et al.*, 1989). As glutamate is known to play an important role in the

normal development of the vertebrate brain (Danbolt, 2001), and since glutamate levels in the honeybee brain also change with age (Fuchs *et al.*, 1989), it would be interesting to investigate the effect of JH on honeybee genes related to glutamergic transmission. The administration of memantine (see Chapter 2) has also been found to regulate the expression of genes that might be involved in neuroprotection (Marvanová *et al.*, 2001), as well as a host of other genes, suggesting a broad-spectrum mode of action for this compound (Marvanová *et al.*, 2004). Finally, recent efforts to use caffeine as a therapeutic agent for sufferers of Parkinson Disease (PD) have uncovered surprising connections between the adenosinergic and the glutamergic signalling pathways in the mammalian brain. For instance, the blockade of adenosine A1 receptors by caffeine leads to an increase in extracellular levels of dopamine and glutamate in the shell of the nucleus accumbens (Solinas *et al.*, 2002), while adenosine A_{2A} receptor mRNA expression is increased in the rat striatum and nucleus accumbens following the administration of memantine (Marvanová and Wong, 2004). In addition, the simultaneous blockade of adenosine A_{2A} and metabotropic glutamate receptors seems to provide a full and immediate recovery of the motor deficits associated with rat models of PD. Such intriguing correspondences between the two receptor systems makes it pertinent to enquire into the effects of caffeine administration on the expression levels of glutamate transporter and receptor genes in the honeybee brain.

6.3 Methods

6.3.1 Organism

Individual frames of brood comb were removed from an experimental hive and placed in an incubator maintained at a constant 32°C. Newly emerged bees were collected from these frames everyday, thus ensuring that the experiments were carried out only on bees of known ages.

6.3.2 Drug administration

Memantine, glutamate and aspartate were mixed in honey to attain final concentrations of 10 mM. Newly-emerged worker bees were fed on this honey for 48 hours (while being reared in the incubator) - control bees were fed on pure honey for the same amount of time. Bees were frozen in liquid N_2 at the end of the treatment period.

2 μL of methoprene and Juvenile Hormone (JH) and caffeine dissolved in di-methyl formamide (dMF) to a concentration of 100 mM were topically applied to the thorax of newly-emerged foragers in three separate treatment groups. Control bees were administered 2 μL of dMF. These bees were also reared in the incubator for 48 hours, before being frozen in liquid N_2.

6.3.3 RNA extraction

Whole brains were dissected out of the frozen bees' heads over a bed of dry ice. Brains were kept frozen on dry ice until the time of further processing. Total RNA was extracted from brain tissue using the Trizol (Invitrogen) reagent.

Five brains from each treatment were pooled for RNA extraction. The tissue was first placed in 1.5 mL eppendorf tubes, and briefly macerated using a pestle. 100 μL of Trizol was added to the tissue, which was further homogenised mechanically. 400 μL more of Trizol was then added to the homogenate, which was then incubated at 37°C for 10 minutes. Following incubation, 100μL of chloroform was added to each tube of homogenate, shaken gently, and spun in a rotary centrifuge at 10,000 rpm for 10 minutes. The resulting top, aqueous phase was transferred to another eppendorf tube, to which was further added 250 μL of isopropyl alcohol. This new mixture was spun in a rotary centrifuge at 13,000 rpm for a further 15 minutes. The colourless RNA pellet now deposited at the bottom of the eppendorf tube was retained, while all the supernatant was discarded. The pellet was then washed twice in 600 μL of 75% ethanol. The pellet was stored in 75% ethanol at -70°C until the next step.

6.3.4 Reverse transcription

The reverse transcription reaction was carried out on the total RNA samples, in a 20μL reaction volume, and using SUPERSCRIPT II RNase H^- Reverse Transcriptase (GibcoBRL). Each RNA sample was dissolved in 11.5 μL of distilled water, to which was added 0.5 μL of oligo-dT primer 5'TTTTTTTTTTTTTTTTTTTTMN (M=ACG, N=ACGT) (PROLIGO Australia). A metabotropic glutamate receptor gene-specific reverse primer (5'GAA GAG CGT TGT GGC GTT CA) was used to generate cDNA in experiments where this gene was being investigated, due to its normally low expression levels. This mixture was incubated at 70°C for two minutes, then immediately transferred to

an ice bath, to allow the primer to bind to mRNA in the sample. A reaction mixture was made up for each RNA sample, containing 4 μL of 5X MMLV Reverse Transcription Buffer (Promega), 2 μL of 10 mM dNTP mix (10 mM each of dATP, dCTP, dGTP and dTTP), and 1μL of RNaseOUT Recombinant Ribonuclease Inhibitor (40 units/μL). The 12 μL of primer-RNA mixture was added to this reaction mixture, and incubated at 42°C for 2 minutes. 1μL (200 units) of SUPERSCRIPT II was then added to the mixture, and the reaction incubated at 42°C for 50 minutes. The reaction was terminated, and the resulting cDNA diluted by the addition of 30μL of Tricine EDTA buffer.

6.3.5 Real-Time PCR

Primers for the target genes metabotropic glutamate receptor (forward, 5' CTA CGT CTC GAC CGT CTA; reverse, 5' ATG TCG TCG AAC ATG CGA TC), NMDA receptor (forward, 5' GGA CAG TAC CAC CAT ACT CA; reverse, 5' GGC CAT CTG TAT CCG AAC TA), glutamate transporter (forward, 5' ACG GTC AGT TTC ACA GCT A; reverse, 5' TCG AAT CAG GAC CTC GAT CA) and s8 (forward, 5'ACG AGG TGC GAA ACT GAC TGA; reverse, 5'GCA CTG TCC AGG TCT ACT CGA) (all from PROLIGO Australia) were generated for use in RT-PCR. S8 (encoding a ribosomal protein) was chosen as a reference gene, as its expression level was found to be constant over a wide range of treatments. A mastermix of the following reaction components was prepared for a final reaction volume of 20 μL: 2 μL 10X PCR buffer, 1.6 μL $MgCl_2$ (4mM), 0.4 μL (0.4 mM), 1μL Syber Green II, 0.2 μL Taq polymerase (1 unit), 11.8 μL distilled water and 1μL each of forward and reverse primer (1 μM). The mastermix was loaded into

real-time RT-PCR tubes (Corbett Research), which were placed on ice. 1μL of template was then carefully pipetted into each tube. Primer efficiency was tested on three different concentrations of cDNA template, namely undiluted, 10X diluted and 100X diluted. Experimental PCR runs to compare treated vs. control bees were carried out on undiluted cDNA. The following PCR cycling protocol was used on the Rotorgene 3000 (Corbett Research), with a single fluorescence measurement: denaturing at 94°C for 2 minutes, then amplification and quantification program repeated 40 times (94°C for 25 seconds, 60°C for 30 seconds, 72°C for 100 seconds with a fluorescence measurement), and finally a melting curve program (60-99°C with a heating rate of 0.2°C per second, and fluorescence measurements being taken at every °C interval).

6.3.6 Data analysis

Real-time PCR data were analysed following the method proposed by Pfaffl (2001). Efficiencies were calculated for all four genes of interest – these efficiency values were then used to calculate relative changes in gene expression between tissues from treated and control bees.

6.4 Results

6.4.1 Primer efficiency and specificity

The primer pairs for all four genes of interest were found to have high real-time PCR efficiency rates, ranging from 1.92 to 2 (Fig. 6.1). The specificity of the amplification products was tested by gel electrophoresis: only single

products with expected lengths could be detected in each case. The melt curves generated by the Rotorgene 3000 analysis program also indicated single products for each of the primer pairs, and with the following melting temperatures: NMDA receptor, 82.6°C; glutamate transporter, 85.7°C; metabotropic glutamate receptor, 92.1°C; s8, 78.6°C.

6.4.2 Treatment-induced changes in expression

The expression of the s8 ribosomal protein gene in the honeybee brain was found to be constant under the conditions being tested, and was therefore used as a reference gene, against which all other gene expression changes were normalised. Juvenile hormone (JH) and methoprene-treated bees showed marked decreases (2.3 to 2.9-fold) in the expression of the metabotropic glutamate receptor gene, and smaller increases (1.4 to 1.5-fold) in the expression of the glutamate transporter gene (Fig. 6.2). The metabotropic glutamate receptor gene was also downregulated by in the case of memantine-treated bees, as was the glutamate transporter gene. The NMDA receptor gene was downregulated more than 1.5-fold in the case of glutamate-treated bees; aspartate-treated bees, in contrast showed an upregulation of both glutamate transporter and NMDA receptor genes. Caffeine treatment did not induce any significant changes in the expression of the genes being tested (Fig. 6.2).

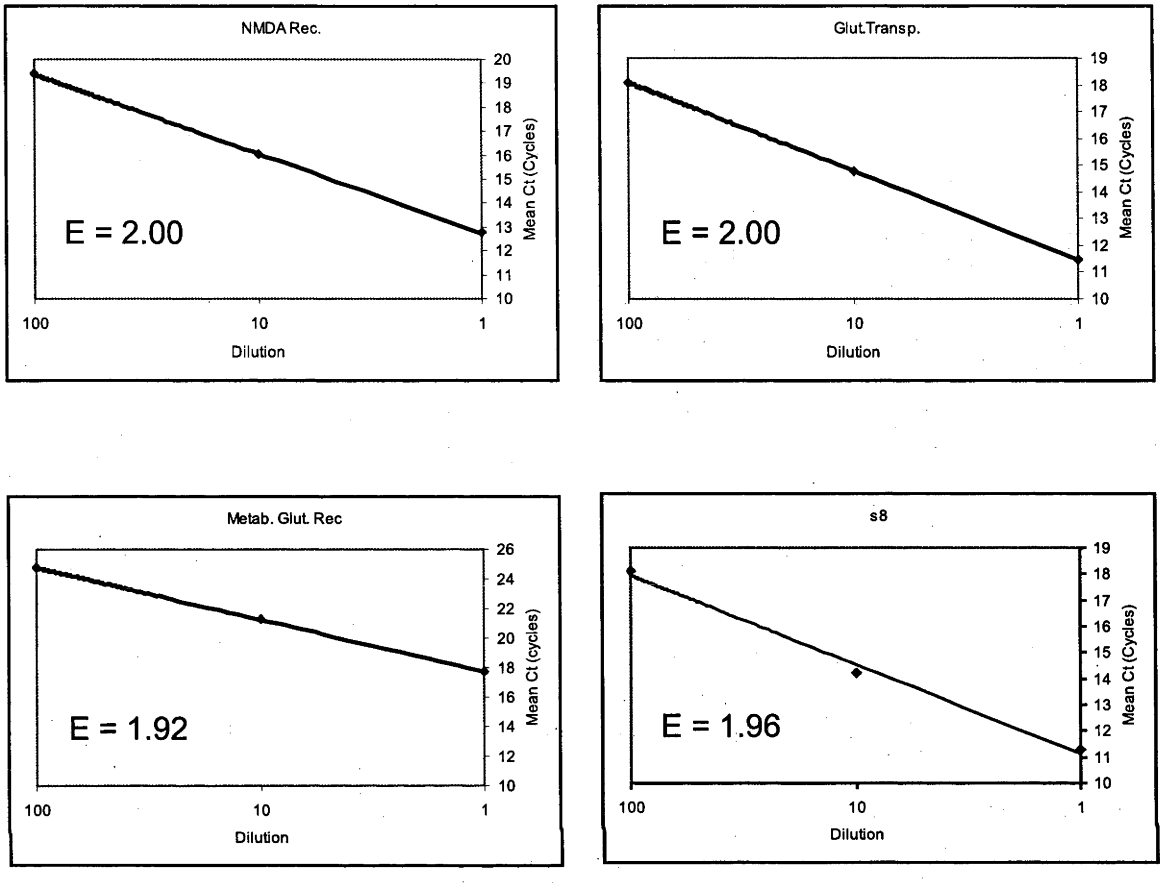
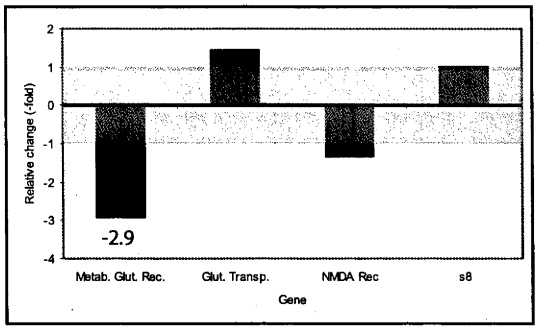
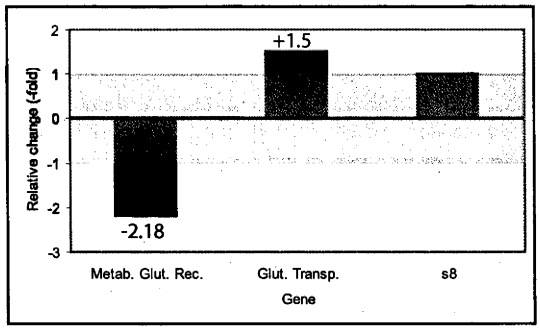


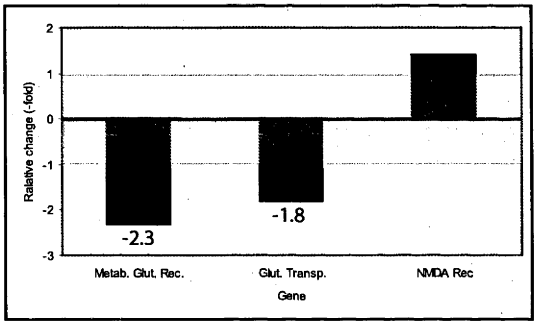
Fig. 6.1. Real-time RT-PCR efficiencies for the four genes of interest, namely NMDA receptor, glutamate transporter, metabotropic glutamate receptor and the reference gene s8. The graphs show the mean Ct values (the mean number of cycles at which the flourescence signal crosses and arbitrary threshold) for three dilutions of the cDNA template. $E = 10^{(-1/slope)}$ = amplification efficiency of the primers.



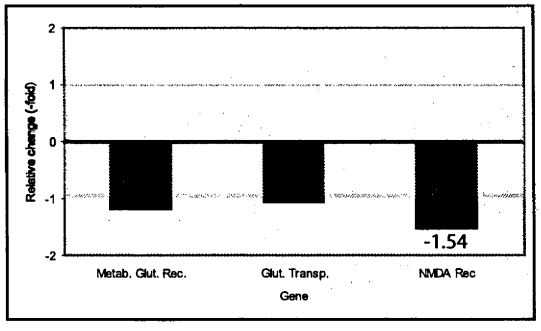
a. Topical juvenile hormone in dMF application



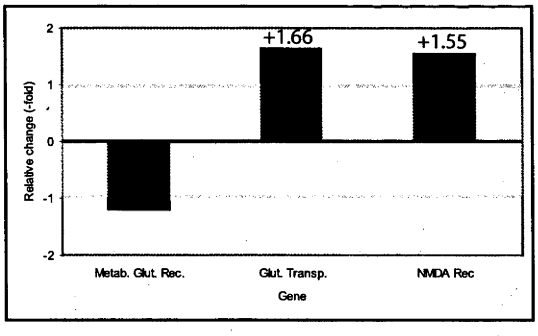
b. Topical methoprene in dMF application



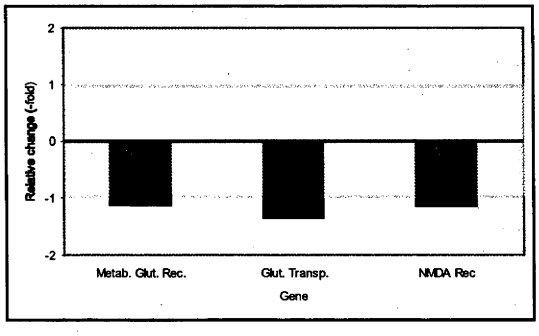
c. Feeding 10 mM memantine in honey



d. Feeding 10 mM glutamate in honey



e. Feeding 10 mM aspartate in honey



f. Topical 100 mM caffeine in dMF

Fig. 6.2. The effect of various pharmacological treatments on the expression of metabotropic glutamate receptor, NMDA receptor and glutamate transporter, as determined by Real-Time RT-PCR. s8 is the reference gene (6.2 a and b), and is assumed to remain constant for these treatments (relative change = 1). Numbers above columns indicate changes of at least 1.5-fold, while the shaded area between +1 and -1 on the y-axis represents a region of unchanged gene expression.

6.5 Discussion

The present study demonstrates the utility of real-time RT-PCR in the detection of changes in the expression levels of selected genes of interest in the honeybee, for which appropriate primers can be designed. The concentrations of the various mastermix components, as well as the cycling program can be easily optimised to enable high and reproducible primer efficiencies. It also demonstrates that the s8 ribosomal protein gene is a good candidate reference gene, whose expression levels show negligible change across a wide range of pharmacological treatments. There are, however, certain experimental conditions, where s8 would not be suitable as a reference gene. This is true in the case of developmental studies, where age-related gene expression levels are being investigated: the expression level of s8 in the honeybee brain has been found to show a large ontogenetic variation (Maleszka *et al.*, unpublished results). Finally, it can be seen that real-time RT-PCR can be successfully used for genes, whose normal expression levels are radically different: the metabotropic glutamate receptor and s8, for instance, have close to a 100-fold difference in their normal expression levels (Fig. 6.2).

The metabotropic glutamate receptor showed the most change in expression level out of all the genes assayed, exhibiting a >2-fold downregulation in bees treated with juvenile hormone (JH), methoprene and memantine. JH or methoprene administration is known to have a wide range of effects on young worker bees, including precocious foraging (Robinson, 1985), the enhancement of short-term olfactory associative memory (Maleszka and Helliwell, 2001) and a

reduction in the behavioural threshold sensitivity to alarm pheromones (Robinson, 1987) at the behavioural level, as well as a significantly larger volume of neuropile in the mushroom bodies and a smaller Kenyon cell somal region (Withers *et al.,* 1994) at the neuroanatomical level. There is growing evidence that JH acts as a 'behavioural pacemaker' in the honeybee (Robinson and Vargo, 1997), and it has been suggested that the molecule might exert its effect on the nervous system by accelerating developmental processes (Maleszka and Helliwell, 2001). The observed decrease in metabotropic glutamate receptor expression brought about by JH and methoprene could be explained in this context. It is possible that the metabotropic glutamate receptor gene is highly expressed in the worker brain soon after emergence, and that the expression level of this gene declines with advancing age. A boost in the rate of nervous system development brought about by JH would lead to the treated bees' having much lower expression levels than the untreated controls. The expression of several genes involved in glutamergic transmission in both vertebrates (Myers *et al.*, 1999) and invertebrates (Soustelle *et al.*, 2002) is developmentally regulated, and the expression of the metabotropic receptor gene mGluR5 in rat hippocampal astrocytes has been found to decline with age (Cai *et al.*, 2000).

The anti-Alzheimer Disease (AD) drug memantine has received considerable attention within the last few years, for its role as a medium affinity NMDA receptor antagonist. Although the link between abnormal glutamergic transmission and AD remains tenuous (Lipton and Rosenberg, 1994), clinical studies have shown positive effects of memantine on patients suffering from moderate to severe AD (Reisberg *et al.*, 2003). The change in metabotropic

glutamate receptor expression reported in the present study might simply reflect the wide-ranging nature of the drug's effects on gene expression in general. Memantine has been shown in vertebrate models to regulate the expression of genes as functionally diverse as regenerating liver inhibitory factor-1 (RL/IF-1), Na+/K+ transporting ATPase 2-beta and adenosine A_{2A} receptor (Maranová *et al.*, 2004; Maranová and Wong, 2004). The downregulation of glutamate transporter, however, might be a consequence of the neuroprotective effect of memantine on NMDA receptors in the bee brain. The upregulation of excitatory amino acid transporter genes has been reported in brain tissue from patients suffering from a range of glutamergic transmission-related neurological disorders, such as autism, schizophrenia and motor neurone disease (Purcell *et al.*, 2001; Smith *et al.*, 2001; Banner *et al.*, 2002). The blocking of NMDA receptors by memantine, and subsequent decreases in NMDA excitation could be responsible for the observed reversal of such trends.

The administration of both glutamate and aspartate induced interesting gene expression changes in the honeybee brain. L-glutamate and L-aspartate are structurally similar (see Fig. 2.1), and the five 'high-affinity' glutamate transporters that have been cloned so far catalyze Na^+- and K^+-coupled transport of L-glutamate as well as L- and D-aspartate (Danbolt, 2001). It is therefore surprising, that glutamate and aspartate should induce such different changes in the expression of both the glutamate transporter and the NMDA receptor. The downregulation of the NMDA receptor by L-glutamate is to be expected: increased ambient concentrations of L-glutamate have been shown to cause a similar downregulation of NMDAR subunits in primary rat cerebellar granule

neurons (Cebers *et al.*, 2001). The aspartate-induced changes, however, are more difficult to explain, and may indicate differential actions of L-glutamate and L-aspartate in the honeybee brain.

Real-time RT-PCR is therefore a powerful technique for the study of differential gene expression in the honeybee. Clearly, much more research needs to be carried out on age-related and treatment-induced changes, before a clear understanding of the complex interactions between development, behaviour and gene expression can be had.

7. Chapter 7: General discussion

7.1 Vertebrate-like effects of drug treatment in honeybees

7.1.1 Glutamate in the honeybee CNS and memory

The use of targeted drugs such as memantine, L-*trans*-2,4-PDC and MK-801 in Chapter 2 revealed that a disruption of the glutamergic signalling pathways in the honeybee CNS could lead to a reduction in the recall of long-term olfactory associative memory. This result lends further support to the idea that L-glutamate might play as important a role in the insect CNS, as it has long been known to in vertebrates. The importance of glutamate in the vertebrate CNS cannot be understated – it is the major excitatory neurotransmitter in the CNS, and is vital to the normal development of the CNS, being involved in processes such as synaptic plasticity and long-term potentiation (Danbolt, 2001). In addition, the NMDA receptor is considered to be a 'classic' learning and memory receptor, due to its requiring a simultaneous depolarisation as well as a ligand (*e.g.* L-glutamate). Until very recently, the status and role of L-glutamate in the insect CNS was practically unknown. The use of the glutamate transporter blocker L-*trans*-2,4-PDC gave one of the first clues to the function of this neurotransmitter in the learning and memory pathways of the honeybee brain (Maleszka *et al.*, 2000). Two metabotropic glutamate receptor subtypes were also recently cloned from the

brain of the honeybee, and were found to be expressed, like the glutamate transporter AmEAAT (Kucharski *et al.*, 2000), in the Kenyon cells of the mushroom bodies (Funada *et al.*, 2004). This further suggests a role for glutamate in higher cognitive functions like learning and memory. The results described in Chapter 2 support the conclusions drawn by Maleszka *et al.* (2000), and show that other drugs, which specifically target the NMDA receptor in vertebrates, have behavioural effects in honeybees similar to those observed in rats, mice and even humans.

The choice of glutamergic drugs used in this study is significant. MK-801, the high-affinity NMDA antagonist, was first developed as an anti-Alzheimer drug. In spite of promising results *in vitro*, however, the drug yielded poor clinical results due to the blocking of normal physiological activity, and the resulting severe side effects, such as hallucinations, ataxia and memory loss (Farlow, 2004). Consistent with this, the loss of long-term olfactory memory was certainly observed in the honeybees treated with this drug prior to training and testing. Memantine, on the other hand, is a promising new anti-Alzheimer drug, which, in recent clinical trials, has been shown to be useful in treating patients with moderate to severe AD, while increasing their autonomy and being clinically well tolerated (Parsons et al., 1999; Palmer and Widzowski, 2000; Rive *et al.*, 2004). At the receptor level, memantine is a medium-affinity antagonist of the NMDAR. By preferentially blocking open channels (Parsons et al., 1999), memantine plays a neuroprotective role in situations where the overstimulation of NMDARs would normally result in excitotoxicity and the eventual death of postsynaptic neuron (Lipton and Rosenberg, 1994; Danbolt, 2001). Regardless of the clinical uses of

memantine, the restoration of the recall of long-term memory in bees treated with L-*trans*-2,4-PDC is strong evidence in favour of the involvement of glutamate in learning and memory in this insect. Based on these findings, a likely scenario would be an abnormal elevation in L-glutamate in the synaptic cleft, brought about by the blocking of glutamate transporter proteins by L-*trans*-2,4-PDC. The resulting overstimulation of NMDAR on the postsynaptic neuron would induce excitotoxicity, and impair the recall of long-term memory. The administration of memantine under such conditions would have the effect of temporarily blocking the open NMDA channels, thereby reversing the effect of L-*trans*-2,4-PDC, and facilitating recall.

Three different glutamergic drugs (two of which specifically target NMDAR) with three different modes of action were used in this study, and were found to produce behavioural changes in the honeybee similar to those observed in vertebrates. These results speak strongly in favour of a learning and memory-related glutamergic pathway in the honeybee brain, and perhaps in the CNS of other insect species as well.

7.1.2 Caffeine and arousal

Strikingly human-like motivation-enhancing effects were observed in caffeine-treated bees used in the experiments reported in Chapter 5. The range of behavioural and cognitive changes brought about by the drug was as large and diverse as that reported from studies on humans – nevertheless, there was a clear enhancement of the arousal state of treated bees, as evidenced by the increased frequency of visits to the feeder, independent of the experimental paradigm.

Caffeine improved performance in a DMTS task, and has been shown in a previous experiment to dramatically accelerate the development of young worker bees, allowing them to learn an olfactory associative task as early as 3 days post-emergence (control bees could only learn the task at 6 days; Maleszka *et al.*, unpublished data). In contrast, the results of the dance experiment showed that caffeine also suppresses the probability of foraging and dancing. This is probably a result of the high concentration of drug administered to the experimental bees, and represents a toxic effect that inhibits various behaviours. While the dance experiment needs to be repeated with a lower concentration of caffeine, it is noteworthy that similar negative effects have also been reported from studies on vertebrates (Lorist and Tops, 2003; Howell and Landrum, 1994).

Although the drugs administered in this thesis were developed specifically for vertebrate nervous systems, they have been shown to have largely the same effects in honeybees as in vertebrates. While there are clearly differences in the gross anatomical organisation of the insect and vertebrate nervous systems (Menzel and Giurfa, 2001), it is really the frequent similarities in the biochemistry and cognitive abilities of the two groups that are most striking (Kandel and Abel, 1995; Bicker, 1999; Müller, 2000; Giurfa, 2003). Indeed, it has been shown that the developmental genetic mechanisms in vertebrate and invertebrate brains are highly conserved, with homeotic (*Hox*) genes being expressed in a virtually colinear anteroposterior pattern in the developing posterior brain of insects and mammals (Kammermeier and Reichert, 2001). It is on the basis of such genetic similarity that a pre-Cambrian divergence between the insect and vertebrate lineages has been proposed (Gu, 1998; Kammermeier and Reichert, 2001). The

recent surge of information on the (vertebrate-like) cognitive abilities of the honeybee, as well as the pharmacological and behavioural data presented in this thesis, strongly suggest the existence of common learning and memory-related signalling pathways between the two lineages.

7.2 Punishing stimuli in honeybee learning paradigms

The olfactory learning experiments discussed above made use of a training protocol that included both rewarding as well as aversive stimuli. The experiments described in Chapter 3 were carried out to systematically compare the learning performance of bees trained to form associations with only a rewarding US, versus that of bees trained with both a rewarding and an aversive US. Two associative learning paradigms were investigated, namely olfactory associative learning using the PER paradigm, and visual associative learning using a Y–maze. The motivation for carrying out these experiments was the observation that bees undergoing training in a y-maze with only a reward would frequently and repeatedly (sometimes more than 10 times) make the same error. It was reasoned that since the making of an incorrect choice merely resulted in a bee's having to attempt the task again, there was no strong incentive for the bee to learn to avoid the wrong pattern. Similarly, the vast majority of published studies on the PER paradigm tend to make use of a single CS-US pairing (usually an odour with a reward of sugar solution), which often results in a very high memory retention score (80-90%) (see Table 1.1). In addition, these studies use as their

experimental animals adult foragers collected at a feeder: these bees would include individuals from a very wide range of ages.

Bees that were trained in a Y-maze with both rewarding and aversive stimuli were found to have a much steeper learning curve than bees trained with only a rewarding stimulus. They were able to learn the visual association task much faster, and also made far fewer repeat errors than their reward-only hivemates. The inclusion of an aversive stimulus clearly made an incorrect choice more costly. The results also show that adult foragers can learn an aversive stimulus in the context of a visual association with ease, much like the findings of Smith *et al.*, (1991) who reported a similar result, but using a PER paradigm. The bees trained in the PER paradigm in the current study, were, however, only 7 days old at the time of training, and 8 days old when tested. While these bees were able to successfully learn a rewarding olfactory association, the scores in the case of the aversive association were much lower. This suggests that while it is a relatively easy matter for bees of any age to learn about a rewarding stimulus, it is only older bees that can learn to avoid a noxious stimulus to any great degree. Young nurse bees who remain within the safety of the hive might rarely, if ever, have to encounter a noxious stimulus. Foragers, on the other hand, are exposed to a much more challenging, and often dangerous, world, filled with potentially harmful stimuli that the bee would have to learn to avoid. The findings reported in Chapter 3 are probably not all that surprising when considered from such a behavioural-ecological point of view.

These findings have important implications for future research on associative learning in the bee. While the inclusion of an aversive stimulus in a

setting making use of free-flying adult foragers might merely accelerate the acquisition of the task at hand, exclusion from training regimes such as the PER paradigm could lead one to overestimate the learning capabilities of one's subjects. Furthermore, these findings highlight the need for the use of single-age bees (or at least bees of known ages) in learning experiments.

7.3 Age and the waggle dance

It had been suspected that age might have been responsible for the aberrant waggle dances that contained high proportions of 'non-waggle' loops, as was reported in Chapter 4. The bees used in the tunnel experiments were a random subset of the forager population of an observation hive, and would therefore have included bees from a wide range of ages. This hypothesis was not supported by the results from Chapter 5, where no major differences in either the distance or the direction being signalled were found with increasing age. This may merely be a result of low bee numbers during the early days of observation, as the trend in waggle duration during the final four days was similar to that reported by an earlier study (Schweiger, 1958). Although this experiment will clearly have to be repeated, it does not offer a satisfactory explanation for the non-waggle loops performed by bees in the tunnel experiments. Bees of all ages performed dances with a high proportion of waggle loops, making age an unlikely reason for the peculiar dances elicited by the tunnel bees. An alternative explanation is that the foragers that were trained to fly in the experimental tunnels were affected by the

contradiction between the high optic flow signals being received by the visual system, and other, visual (such as external landmarks) or non-visual cues (such as flight duration) that indicated that they had only flown a short distance. In essence, the tunnel bees were not completely fooled by the optic flow: the abnormal dance reflects the conflicting sensory information imposed upon them by the experimental apparatus.

It is important to note that some parameters of foraging and dance behaviour *were* seen to depend on the age of the bees: these included the probability of foraging, foraging frequency and the probability of dancing. However, an astonishing amount of variation was observed in propensity of experimental bees to dance and forage. It is a well known phenomenon that worker bees vary greatly in the age at which they first begin foraging (Winston, 1987). A similar pattern was observed in the present study, along with a large variation in the age at which foragers started dancing, in spite of having made repeated visits to the feeder. Most of the control bees only performed their first dance during the last 3 days of observation (when they were 20-22 days old), while one started dancing as early as day 13 of her life. In addition, the average control bee had to make approximately 30 flights to the food source before she started dancing at all. The process of starting to dance therefore seems to be a long and protracted one, where a bee must first follow a dance at the hive, find the food source being indicated, and then fly that route several times before performing a dance of her own. These results seem to favour the hypothesis that the dance behaviour is at least partly a learnt one, and reveal some intriguing similarities between the acquisition of the waggle dance by young foragers and the acquisition

of language by human children. Firstly, just as language perception in a human child always precedes language production (Jusczyk, 1997), so also can the young honeybee 'read' the dances of more experienced foragers well before she performs her own, first dance. Secondly, the acquisition of both symbolic systems seems to be interactive, *i.e.* a young forager seems to have to follow one (or more likely several) dancer(s) at the hive, find the indicated food source, and fly the route several times before being able to accurately signal the food source through her own dance. Similarly, a human child would have to use his or her language in meaningful ways to become proficient in it; simply having the noise of a language around one does not allow one to learn it (Bruner, 1975).

Most surprising of all was the finding that approximately half of the total dances filmed and analysed during the observation period were performed by only 6 of a total of 39 (~15%) experimental bees. Half the experimental bees that were seen to dance performed only 4 or less dances each, while a handful of bees (~5) were not seen to dance at all, in spite of repeated trips to the feeder. Such a dichotomy in the tendency to dance suggests a role for genetic variation in determining the behavioural patterns of foragers later in adult life. Gene expression profiles have been found to predict behavioural plasticity in honeybees, in terms of the age at which a nurse bee makes the transition to foraging behaviour (Whitfield *et al.*, 2003). It is therefore conceivable for other ontogenetic landmarks, such as the onset of dancing, to be dependant on the gene expression profile of individual bees. It would also not be surprising if consistent gene expression patterns were to exist, that could reliably be used to distinguish 'good'

foragers from 'bad' foragers, as well as 'good' dancers from 'bad' ones, on the basis of visit and dance frequencies.

7.4 Gene expression changes

Real-time RT-PCR was found to be a convenient and reliable means of investigating changes in the expression of individual genes. Requiring only a small amount of cDNA as template (and hence small amounts of tissue), this method can be used to investigate expression changes in, for example, the entire brain or even part of the brain of an individual honeybee. The pharmacological treatments applied in Chapters 2 and 5 were found to induce modest changes in the expression of genes associated with glutamergic signalling pathways, in keeping with their known modes of action and observed behavioural effects. Such a combination of methods would appear to be a profitable basis for the planning of future experiments, wherein gene expression could be investigated, for instance, before and after a learning paradigm, or to compare individuals of differing ages or behavioural tendencies.

7.5 Concluding remarks

The honeybee, while being among the best studied of all invertebrate species, has yet managed to keep hidden some of the most vital and intriguing aspects of its comportment, physiology and molecular machinery. The honeybee has been shown to be capable of amazing (for an insect) feats of learning, while

the neuronal pathways that facilitate this learning remain largely to be explored. The bee's dance communication system is unrivalled among all animal taxa outside the vertebrates, and yet the mechanism by which foraging bees convert the location of a food source into a series of stereotyped movements remains unclear. This thesis goes some way towards providing solutions to these mysteries. It presents further evidence for the involvement of the neurotransmitter L-glutamate in the recall of olfactory associative memory, and shows that learning protocols produce more reliable results with the inclusion of an aversive stimulus pair along with the rewarding one. Finally, it shows that certain aspects of honeybee foraging and dance behaviour are ontogenetically regulated, and that caffeine can have disruptive effects on several of these aspects, at least at the concentration tested. The approach of combining behavioural, pharmacological and molecular techniques is clearly a fruitful one, which should yield important, meaningful results regardless of the paradigm being employed.

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Appendix: Publications resulting from work done during the PhD

- Aung Si, M. V. Srinivasan and S. W. Zhang (2003), Honeybee navigation: Properties of the visually driven 'odometer'. *J. Exp. Biol.*, **206 (8):** 1265-1273.
- Aung Si, P. Helliwell and R. Maleszka (2004), Effects of NMDA receptor antagonists on olfactory learning and memory in the honeybee (*Apis mellifera). Pharm. Biochem. Behav.*, **77:** 191-197.
- J. Tautz, S. W. Zhang, J. Spaethe, A. Brockmann, Aung Si and M. V. Srinivasan (2004), Honeybee odometry: performance in varying natural terrain. *PLOS Biology*, **2 (7):** 915-923.